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THE

BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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CONTENTS

No. 1, August, 1975

Annual Report of the Marine Biological Laboratory 1

ANDERSON, PETER A. V. AND JAMES F. CASE
Electrical activity associated with luminescence and other colonial behavior in the pennatulid *Renilla köllikeri* 80

BEITINGER, THOMAS L.
Diel activity rhythms and thermoregulatory behavior of bluegill in response to unnatural photoperiods 96

BLAKE, JAMES A. AND KEITH H. WOODWICK
Reproduction and larval development of *Pseudopolydora paucibranchiata* (Okuda) and *Pseudopolydora kempi* (Southern) (Polychaeta: Spionidae) 109

BROWN, JUDITH A.
Sidereal-day variation in spontaneous activity of the mouse, *Mus musculus* 128

BROWN, STEPHEN C.
Biomechanics of water-pumping by *Chaetopterus variopedatus* Renier. Skeletomusculature and kinematics 136

CHIPPENDALE, G. M. AND C.-M. YIN
Reappraisal of proctodone involvement in the hormonal regulation of larval diapause 151

FINGERMAN, MILTON, SUE W. FINGERMAN AND DREW T. LAMBERT
Colchicine, cytochalasin B, and pigment movements in ovarian and integumentary erythrophores of the prawn, *Palaemonetes vulgaris* 165

LYERLA, TIMOTHY A., JOANN H. LYERLA AND MARILYN FISHER
Pigmentation in the orange tunicate, *Ecteinascidia turbinata* 178

MAZURKIEWICZ, MICHAEL
Larval development and habits of *Laeonereis culveri* (Webster) (Polychaeta: Nereidae) 186

MITCHELL, K. A.
An analysis of shell occupation by two sympatric species of hermit crab. I. Ecological factors 205

NIJHOUT, H. FREDERIK
A threshold size for metamorphosis in the tobacco hornworm, *Manduca sexta* (L.) 214

RAVINDRANATH, M. H.
Effects of hydrogen ion concentration on the morphology of hemocytes of the mole-crab *Emerita asiatica* 226

SPENCER, ANDREW N.
Behavior and electrical activity in the hydrozoan *Proboscidactyla flavicirrata* (Brandt). II. The medusa 236

VIA, SARA E., AND RICHARD B. FORWARD, JR.
The ontogeny and spectral sensitivity of polarotaxis in larvae of the crab *Rhithropanopeus harrisi* (Gould) 251

No. 2, October, 1975

Bone, Q. and G. O. Mackie
Skin impulses and locomotion in *Oikopleura* (Tunicata: Larvacea) 267
Calder, Dale R.
Biotic census of Cape Cod Bay: hydroids 287
Fyhn, Unni E. H. and John D. Costlow
Tissue cultures of cirripeds 316
Hamby, Robert Jay
Heat effects on a marine snail 331
Hassall, Mark and J. B. Jennings
Adaptive features of gut structure and digestive physiology in the terrestrial isopod *Philoscia muscorum* (Scopoli) 1763 348
Jones, Jack Colvard
Nonheterosexual behavior in mosquitoes 365
Lund, William A., Jr. and Barton C. Marcy, Jr.
Early development of the grubby, *Myoxocephalus aenaeus* (Mitchill) ... 373
O'Meara, George F. and John D. Edman
Autogenous egg production in the salt-marsh mosquito, *Aedes taeniorhynchus* 384
Stephens, Grover C.
Uptake of naturally occurring primary amines by marine annelids 397
Stross, R. G.
Causes of daily rhythms in photosynthetic rates of phytoplankton. II. Phosphate control of expression in tundra ponds 408
Abstracts of papers presented at the Marine Biological Laboratory 419

No. 3, December, 1975

Azariah, Jayapaul, M. Mohamed Ismail, and M. Ahmed Najib
Investigation on the ecology and respiratory responses of the Hemichordate *Ptychodera flava* to tidal cycles and salinity changes 455
Castrucci, Ana Maria De L., and Erasmo G. Mendes
Ultrastructure of the pigmentary system and chromatophorotropic activity in land isopods 467
Del Pino, Eugenia M., Maria L. Galarza, Carmen M. De Albuja, and A. A. Humphries, Jr.
The maternal pouch and development in the marsupial frog *Gastrotheca riobambae* (Fowler) 480
Egan, W. G., and J. E. Conrad
Summer abundance and ecology of zooplankton in the Gulf Stream 492
Fain, Margery J. and Lynn M. Riddiford
Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L.) 506
Fuzessery, Zoltan M., and James J. Childress
Comparative chemosensitivity to amino acids and their role in the feeding activity of bathypelagic and littoral crustaceans 522
Harris, Larry G.
Studies on the life history of two coral-eating nudibranchs of the genus *Phestilla* 539

NEWTON, W. DONALD
The ultrastructure of the aflagellate spermatozoon of the freshwater turbellarian *Hydrolimax grisea* (Platyhelminthes: Plagiostomidae) 551
NIJHOUT, H. FREDERIK
Dynamics of juvenile hormone action in larvae of the tobacco hornworm, *Manduca sexta* (L.) 568
PECHENIK, JAN A.
The escape of veligers from the egg capsules of *Nassarius obsoletus* and *Nassarius trivittatus* (Gastropoda, Prosobranchia) 580
STEELE, R. DUNBAR
Stages in the life history of a symbiotic zooxanthella in pellets extruded by its host *Aiptasia tagetes* (Duch. and Mich.) (Coelenterata, Anthozoa) 590

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INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. ***Manuscripts.*** Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. ***Tables, Foot-Notes, Figure Legends, etc.*** Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

Vol. 149, No. 1 August, 1975

THE BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY

SEVENTY-SEVENTH REPORT, FOR THE YEAR 1974—EIGHTY-SEVENTH YEAR

I. TRUSTEES AND EXECUTIVE COMMITTEE (AS OF AUGUST, 1974) 1
II. CERTIFICATE OF INCORPORATION 5
III. BYLAWS OF THE CORPORATION 5
IV. REPORT OF THE DIRECTOR 7

Addenda:

1. The Staff 12
2. Investigators, Fellowships, and Students 22
3. Fellows and Scholarships 33
4. Training Programs 34
5. Tabular View of Attendance, 1969–1973 37
6. Institutions Represented 37
7. Friday Evening Lectures 39
8. Members of the Corporation 40

V. REPORT OF THE LIBRARIAN 71
VI. REPORT OF THE TREASURER 72

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II. CERTIFICATE OF INCORPORATION

No. 3170

COMMOMWEALTH OF MASSACHUSETTS

Be it Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth

III. BYLAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

(Revised February 11, 1972)

I. The members of the corporation shall consist of persons elected by the Board of Trustees.

II. The Officers of the Corporation shall consist of a Chairman of the Board of Trustees, President, Director, Treasurer and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and nine Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these bylaws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Massachusetts. Special meetings of the Trustees shall be called by the Chairman, the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-six Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed.

(B) Trustees *ex officio*, who shall be the Chairman, the President, the Director, the Treasurer, and the Clerk.

(C) Trustees *Emeriti*, who shall include any member of the Corporation in good standing who has attained the age of seventy years, or has attained the age of sixty-five and has retired from his home institution, and who has served a full elected term as a regular Trustee, shall be designated Trustee Emeritus for life at the next annual meeting provided he signifies his wish to serve the Laboratory in that capacity. Any regular trustee who qualifies for emeritus status shall continue to serve as Trustee until the next Annual Meeting whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation. The Trustees *ex officio* and *Emeriti* shall have all the rights of the Trustees, except that *Trustees Emeriti* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation. They shall elect a Chairman of the Board of Trustees who shall be elected annually and shall serve until his successor is selected and qualified and who shall also preside at meetings of the Corporation. They shall elect a President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and Vice Chairman of meetings of the Corporation, and who shall be elected annually and shall serve until his successor is selected and qualified. They shall appoint a Director of the Laboratory for a term not to exceed five years, provided the term shall not exceed one year if the candidate has attained the age of 65 years prior to the date of the appointment. They may choose such other officers and agents as they may think best. They may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them except those chosen by the members, at any time. They may fill vacancies occurring in any manner in their own number or in any of the officers. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These bylaws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the bylaws will be acted upon.

RESOLUTIONS ADOPTED BY THE TRUSTEES:

I. RESOLVED:

(A) The Executive Committee is hereby designated to consist of not more than ten members, including the *ex officio* members (Chairman of the Board of Trustees, President, Director and Treasurer); and six additional Trustees, two of whom shall be elected by the Board of Trustees each year, to serve for a three-year term. (August 11, 1967.)

(B) The Chairman of the Board of Trustees shall act as Chairman of the Executive Committee, and the President as Vice Chairman. A majority of the members of the Executive Committee shall constitute a quorum and a majority of those present at any properly held meeting shall determine its action. It shall meet at such times and places and upon such notice and appoint such sub-committees as the Committee shall determine. (August 12, 1966).

(C) The Executive Committee shall have and may exercise all the powers of the Board during the intervals between meetings of the Board of Trustees except those powers specifically withheld from time to time by the Board or by law. (August 16, 1963).

(D) The Executive Committee shall keep appropriate minutes of its meetings, and its action shall be reported to the Board of Trustees. (August 16, 1963).

II. RESOLVED:

The elected members of the Executive Committee be constituted as a standing "Committee for the Nominations of Officers," responsible for making nominations, at each Annual Meeting of the Corporation, and of the Board of Trustees, for candidates to fill each office as the respective terms of office expire (Chairman of the Board, President, Director, Treasurer, and Clerk). (August 16, 1963).

III. RESOLVED:

Any member of the Corporation in good standing who has attained the age of seventy years, or has attained the age of sixty-five and has retired from his home institution, shall automatically be designated a Life Member of the Corporation provided he signifies his wish to retain his membership in the Corporation. Life Members shall not have the right to vote and shall not be subject to the payment of any dues. (February 16, 1973).

IV. REPORT OF THE DIRECTOR

To: The Trustees of the Marine Biological Laboratory

The Friendship Fund Deed of Trust of 1924, established for the benefit of the Marine Biological Laboratory, provides for a Committee of Review to evaluate the work of the Laboratory at ten-year intervals. The Committee that met in Woods Hole on August 15 and 16, 1974 determined that "the Laboratory is completely fulfilling the purposes for which the Friendship Fund was created," and concluded that "the Marine Biological Laboratory is a unique and valuable institution, of quality unmatched elsewhere, and truly a national and not merely a regional asset in American science."

It is the responsibility of the Officers and Trustees, not merely to preserve, but to strengthen this unparalleled resource: to hold fast to the renowned summer research and teaching programs, for as the Committee wrote, ". . . it is precisely the present flux of students and investigators in the summer that generates the uniquely valuable characteristics. . . ," to extend this feature of the Laboratory throughout the year, and to design year-round research programs that will enrich, and be enriched by, the summer programs.

How well have we succeeded in 1974–1975? I shall take up this question under the next three headings.

Summer research and teaching

In their Annual Meetings on August 16, 1974, Corporation Members and Trustees alike voiced concern for the decline in numbers of highly qualified young investigators and students at the Laboratory and urged that no effort be spared in providing attractive, well funded programs designed to kindle renewed interest in this crucial segment of our scientific population. Our response to this need has been the development of a new program for gifted young scientists. It is designed to provide young investigators with the facilities, associations and training needed for them to apply and develop their research abilities, enabling them to take their critical *Steps Toward Independence*, as the program is entitled. Happily, our concern for young investigators and students has been shared by the leaders of the Robert Sterling Clark Foundation, the Jessie Smith Noyes Foundation and the Surdna Foundation, all of which have provided funds for scholarships.

Significant support for training has come from other directions as well. The Alfred P. Sloan Foundation has awarded a three-year grant in support of the summer Neurobiology course, now under the direction of Edward Kravitz and A. O. W. Stretton. These new funds, coupled with the continuing support of the Grass Foundation, shall insure that we will be able to enrich the scientific careers of at least twelve young neurobiologists each summer. The Sloan Foundation was also instrumental in setting up the January Short-Term course in Neurobiology, offered jointly by the Boston University Marine Program at the Laboratory, with Fred Lang, Alan Fein and E. F. MacNichol, Jr. in charge. In this case, the Sloan grant went to BUMP. Still another grant, which was awarded jointly to BUMP and MBL by the Charles Hayden Foundation, provided teaching equipment.

It is reassuring that our programs for young scientists and students merit the support of foundations which are known for their critical and perceptive reviews. There are other measures of vitality in education: one is the ability to maintain quality in the face of change. The year 1974–1975 was a year of change in the leadership of many of our courses. Gifted instructors stepped aside, to be replaced by others no less qualified. I referred earlier to Kravitz and Stretton, who succeeded Michael Bennett and John Dowling. Eric Davidson completed his term in Embryology, as did Robert Josephson in Experimental Invertebrate Zoology and Holger Jannasch in Ecology, to be replaced by David Epel and Tom Humphreys, Michael Greenberg, and Frederick Smith and George Woodwell, respectively.

There are two further significant departures arising out of these changes. As both the Neurobiology and Embryology courses increased their emphasis on the structure and function of cell membranes, the Research Training Program in Excitable Membrane

Biophysics and Physiology was closed. The Program had filled a need for a number of years, most recently under the able direction of David Goldman, and its termination was not universally applauded. The loss of this program, however, is balanced by the gain of a new Program in Microbial Ecology, headed by Holger Jannasch, a healthy outgrowth of the former course in Experimental Microbial Ecology.

Finally, special thanks should be extended to Frank Loewus who relinquished the leadership of the Research Program in Experimental Marine Botany. Jerome Schiff, who also heads the course in Comparative Biology and Biochemistry of Algae, succeeds him.

Year-round teaching

Many students have testified that their summer course experience at MBL was extraordinary and produced a lasting effect on their careers. In the Report of the Director for 1973, I wrote of one of the Laboratory's guiding principles, the early exposure of students to research: "The critical turn of mind fostered by research tempers the student's approach to the evolving base of science and its applications."

Everyone who has worked at the MBL will have his own favorite example of this maxim. A striking new one emerged in the Physiology Course in 1974. Gerald Weissmann of New York University School of Medicine, an instructor in the course, and several colleagues and students joined forces to develop a general method for introducing enzymes into cells lacking them. Those participating were David Bloomgarden, Sylvia Hoffstein, Roberta Kaplan and Charles Cohen, all of NYU; Tucker Collins of Amherst; Avrum Gottleib of McGill and David Nagle of the University of Massachusetts.

The work was presented in Weissmann's lecture on Friday evening, August 23rd, and a complete article appeared in *The Proceedings of the National Academy of Sciences.* They have shown that phagocytes of the smooth dogfish, which contain none of the enzyme, peroxidase, within their lysosomes and thus constitute models for cells genetically deficient in lysosomal enzymes, will take up the enzyme providing it is presented in a specific manner. The peroxidase enzyme must be incorporated into lipid vesicles called "liposomes" which have been coated with heat-aggregated dogfish immunoglobin (antibody). The dogfish were thus "fooled" into treating the reparative enzyme as if it were an invader against which they had mobilized their antibody defenses. They reacted by engulfing it into the very organelles (lysosomes) which lacked the enzyme in the first place. Since over thirty human diseases are characterized by a lack of one or another lysosomal enzyme, this method may prove to be of general use in providing enzymes to human phagocytic cells genetically deficient in them.

This work, which was nominated for the 1974 MBL Award (as were two other student-faculty studies among the eight nominees), is but one example of the challenge facing the year-round teaching programs. All of us agree that the splendid facilities of the Laboratory should be exploited more fully during the winter, but we are concerned lest the quality of the winter offerings falls short of the renowned summer standard.

Thus far I have been reassured by the January Short-Term offerings. Earlier I spoke of the Neurobiology course, one of four offered in 1975, the others being Behavior, Developmental Biology, and the Biosphere. Like Neurobiology, Behavior, headed by Robert Jeanne, was a joint BUMP-MBL effort.

The Biosphere was the first course offered by our own Ecosystems Center, under George Woodwell's direction; and Developmental Biology, last year's "trailblazer," was again headed by L. E. DeLanney, with help from J. R. Whittaker and W. S. Vincent, with the last-named to head the course next year, when all four courses will again be offered.

The more than eighty students who were enrolled in the courses received a rich and varied experience. Many of the special lectures were attended by well over one hundred students, staff and visitors from the Woods Hole scientific community. It was common for lectures at any time of day to draw audiences of more than fifty. Students were encouraged to attend special lectures in the courses other than the course for which they had enrolled. Enthusiasm ran high among both students and staff.

I have one concern about these courses which is shared by G. M. Woodwell, Assistant Director for Education. My original plan in developing these courses was to offer opportunities for students who are in colleges and universities that have a break in January. In these schools undergraduates are expected to seek an unusual experience in education and I have believed that we can offer such an experience at the MBL. There is, however, considerable interest in these courses among graduate students and there is a natural tendency to raise the level of presentation to the graduate level. The Developmental Biology course has in both years been aimed largely at undergraduates. The other three courses offered in this past January tended more toward the graduate level. Woodwell and I agree that an undergraduate focus seems more appropriate in that it avoids the possibility of competition with our own summer graduate programs and strikes more forcefully at a specific role that we should play in supplementing the offerings of colleges and universities. I have asked that the Instruction Committee consider this question in detail during the summer and offer a recommendation. The courses are now advertised by request of several instructors as "advanced undergraduate and beginning graduate level."

I continue to enjoy working with Arthur Humes, under whose guidance the Boston University Marine Program is attracting better students and doing more for them. I hope that, now that it is established that BUMP is not merely viable, but steadily growing in stature, the Program can be expanded, with two or possibly three more staff members in residence. The program is well known, draws students from around the country at both graduate and undergraduate levels, and is an extremely important part of the educational program of the Woods Hole community.

Our association with Boston University took another turn early in 1975, when the Sea Education Association moved its land-based teaching program from Boston to Woods Hole. SEA provides a semester-long course in marine study and practice. Students earn eight hours' credit from Boston University for a six-week academic term taught at MBL and another eight hours' credit for six weeks aboard the schooner *Westward*. The SEA program, which is directed largely toward college sophomores, includes introductions to marine science, nautical science, and "Man and the Sea," treating historical, economic, social and marine policy questions. The Director of the program in Edward C. Monahan, who is known to many members of the Corporation since he has been offering an annual course for University of Michigan students in Woods Hole since the spring of 1971.

These additions to the educational program lend further strength to the Laboratory's year-round activities. In the fall of 1975 we will offer for the first time the possibility of a student's taking "A Year in Science at the MBL." The first courses in this new program will be offered by the staff of the Ecosystems Center around a core course: A Seminar on the Biosphere. Under this program undergraduates and graduate students will be encouraged to come to the Marine Biological Laboratory to arrange special programs of study and research in science using the entire scientific community of Woods Hole in ways that have been tailored especially to the needs of individual students. We expect a small group of students during the fall semester of 1975. This program is being supported by scholarship funds from the Robert Sterling Clark Foundation and the Jessie Smith Noyes Foundation.

Year-round research

The decisions we have made in the development of year-round programs are in the process of being implemented. Two of the three topics we hope to develop are now represented at the MBL with continuing programs: ecology and neurobiology. The Ecosystems Center was opened on January 1, 1975 under the direction of George M. Woodwell, who has been in residence for increasing periods of time through the winter and spring. He will be in Woods Hole full time this summer when he will be joined by Daniel Botkin, currently at Yale, Charles A. S. Hall, currently at Cornell, and a group of distinguished staff. The development of the Center has been made possible by a series of special gifts including initial gifts from the Clowes Fund and the Grass Foundation, a special challenge grant of the Henry L. and Grace Doherty Charitable Foundation and major grants from six other foundations and corporations whose ranks include the Andrew W. Mellon Foundation, the Rowland Foundation, the Charles E. Culpeper Foundation, the Exxon Corporation, the Max C. Fleischmann Foundation and the General Electric Foundation. These awards, together with unrestricted gifts for year-round programs from the Fred Harris Daniels Foundation, the William E. and Bertha E. Schrafft Charitable Trust and the Edwin S. Webster Foundation, and the grants in support of education already mentioned, bring the total given or pledged by private foundations to well over two million dollars.

The initial efforts of the Center have been in the development of the MBL's educational programs, including especially the January course and the year-round program. Two research programs are gathering momentum now as a part of the Center's activities. The question of how estuarine marshes function as units of the surface of the earth is the focus of one of these programs; the effects of chronic disturbance on the structure and function of terrestrial communities is the focus of the second program. Within these two topics separate projects range from studies of segments of the world carbon budget through measurements and interpretation of carbon dioxide fluctuations in the atmosphere, through population models of elephants in Africa, to analyses of gradients of change in forest structure caused by accumulations of toxins. The Center's special emphasis is on the types of research that cannot easily be done without the kinds of special facilities and field opportunities that the Marine Biological Laboratory offers. One special attribute of the Center and the Laboratory is recognized as the thoroughly demonstrated ability of the Laboratory to attract distinguished scientists from other institutions from around the world.

The Laboratory of Sensory Physiology is also gaining additional momentum both through contributions to the Laboratory's summer and winter educational programs and through the gradual additions of staff. Alan Fein was honored recently by being selected as a Sloan Research Fellow for a two-year period beginning in September, 1975. Plans are underway to increase year-round research in neurobiology significantly.

Both year-round research and teaching have been advanced significantly by the time and energy devoted by the Assistant Director for Research Sciences, E. F. MacNichol, Jr., and his colleagues, F. P. Bowles and J. A. Hancock, and Robert Gunning and the Department of Buildings and Grounds to remodelling facilities and upgrading equipment.

MBL Associates and the new Quadrangle

As this report is being written, the new Quadrangle is fast being transformed into a beauty spot. Much of the construction work has been completed and at this very moment in late April trees are being planted. Another three weeks will see the completion of the project.

This remarkable development would not have been possible were it not for the MBL Associates and their tireless President, Peggy Clowes and Treasurer, Rhea Zwilling. In the year beginning in the spring of 1974, the Associates have raised $105,000 for the landscaping project (including $50,000 from the Richard King Mellon Charitable Trusts), and have assured the Laboratory that they will not rest until the balance of monies needed to complete the project are in hand and until there is a significant addition to the endowment to provide for its maintenance.

Losses—and gains

In last year's Report I told of my reliance on the "at large" Officers and Trustees, and spoke of my pleasure at the reapportionment of the Board of Trustees whereby three members of each Trustee class will be "at large" members. Although Richard S. Morse completed his term as a Trustee in 1974, he is continuing to work in behalf of the Laboratory. Three new "at large" members—Prosser Gifford, Ellen Grass and John Kendall—have already begun to take up their new responsibilities. Perhaps the latter statement should be modified in the case of Ellen Grass, who is continuing to work for the Laboratory as she always has, but now more "officially."

February 14, 1975 may go down as a watershed in the Laboratory's history. It was on that date that the Trustees elected the distinguished cell biologist, Keith Roberts Porter, to be the Laboratory's next Director. I count it a special privilege to have been able to set the stage for him.

I am also especially pleased to announce the appointment of Mr. Charles Ossola as Assistant Director for Finance and Development. It has been my good fortune to have worked with him in the past and I look forward to his stewardship. He will work closely with the Director, Treasurer and General Manager in budgeting and grants management and with the President in long-range planning and financial development.

It also gives me special pleasure to note the election of another of our Corporation members, Howard A. Schneiderman, to the National Academy of Sciences. Another member of the Corporation, Eugene Odum, was honored, along with his brother, H. T. Odum, on June 18, 1975 by the award of the Prize of Institut de la Vie, which in 1975 was underwritten by Fondation Electricite de France. The Odums were cited for their pioneering investigations in experimental ecology.

Stability with flux

Although there has been, and will continue to be, considerable turnover within its parts, the Laboratory enjoys stability. We achieve what Rudolph Schoenheimer liked to call *stability with flux*. I touched upon the secret of our success in an earlier report. Perhaps I should add parenthetically that one of the several hazards of being a "Double Director" is that one becomes especially prone to the condition described by David Segal: "To quote himself with consummate ease—Man's earliest incurable disease."

What I wrote in 1972 was this: ". . . not all the moving vigor can flow endlessly from the Director. It needs to be generated and manifested at every level." Our secret is just that—there is a never-ending spring of human energy at the Laboratory—in its dedicated staff, in its Standing Committes, in its investigators in residence, and in its Associates. These springs want to be tapped, need to be tapped. I have been happier in the directorship since I learned that.

1. The Staff

EMBRYOLOGY

I. Instructors

Eric Davidson, Professor of Biology, California Institute of Technology, director of course

Roy Britten, Research Associate of Biology, California Institute of Technology and Staff, Carnegie Institution of Washington, associate director of course

Tom Humphreys, Associate Professor, Kewalo Marine Lab, University of Hawaii

Fotis Kafatos, Professor of Biology, Harvard University

II. CONSULTANT

L. DENNIS SMITH, Professor of Biology, Purdue University

III. ASSISTANT

GLEN GALAU, Graduate Fellow of the California Institute of Technology

IV. LECTURES

ROY J. BRITTEN	DNA sequence organization in eukaryotes
ERIC H. DAVIDSON	Organization of transcribed sequences and the interspersion of structural genes in eukaryotic DNA
TOM HUMPHREYS	RNA synthesis during sea urchin development
FOTIS C. KAFATOS	Programs of specific protein synthesis in the differentiation of insect cells
BRUCE BRANDHORST	Kinetic investigations of the metabolism of RNA and poly (A) in 'mammalian' cells
L. DENNIS SMITH	Genomic activation by hormones
PAUL GROSS	Transcription of histone genes in sea urchin embryos
JAMES E. DARRELL	Molecular characteristics of hnRNA in mammalian cells and its possible role as mRNA precursor
ROBERT PERRY	Synthesis and turnover of polyadenylated RNA's in mammalian cells
RICHARD AXEL	Structure of mammalian chromatin and its transcription *in vitro*
RONALD REEDER	Transcription of chromatin by homologous and heterologous RNA polymerase
ROBERT SCHIMKE	Quantitative studies on purified ovalbumin mRNA and its precursor
CHARLES A. THOMAS	Restriction endonuclease studies on eukaryotic DNA
JOHN O. BISHOP	Sequence organization around the hemoglobin structural gene
DAVID HOGNESS	Sequence organization in *Drosophila* DNA studied with replicated *Drosophila* DNA fragments

PHYSIOLOGY

I. INSTRUCTORS

JOHN J. CEBRA, The Johns Hopkins University, in charge of course
GARY ACKERS, University of Virginia
DENNIS BARRETT, University of Denver
WILLIAM CLEM, University of Florida
DANIEL GOODENOUGH, Harvard University
PIEN-CHIEN HUANG, The Johns Hopkins University School of Hygiene and Public Health
RU-CHIH C. HUANG, The Johns Hopkins University
THOMAS D. POLLARD, Harvard University
DENNIS POWERS, The Johns Hopkins University
ROBERT A. PRENDERGAST, The Johns Hopkins University School of Medicine
RICHARD RODEWALD, University of Virginia
ERIC WEINBERG, The Johns Hopkins University
GERALD WEISSMANN, New York University School of Medicine

II. Assistants

Martha Barrett, University of Denver
Calvina Baumgartner, The Johns Hopkins University

III. Lectures

Daniel Goodenough	The structure of biomembranes: historical perspective
Daniel Goodenough	The structure of biomembranes: current theories
Daniel Goodenough	Structure and function of some intercellular junctions
Gerald Weissmann	Lysosomes and inflammation
Gerald Weissmann	The control of phagocytic and other cellular functions by cyclic nucleotide mediated assembly of microtubules
Gerald Weissmann	Biomembranes and cell lysis
William Clem	Immunologic phenomena
William Clem	Phylogeny of the immune response
Robert Prendergast	Cellular immunity
Robert Prendergast	Lymphocytes and lymphokines: their behavior *in vitro*
Robert Prendergast	Immune pathology
John Cebra	T- and B-lymphocytes: differentiation, final maturation and interaction
Dennis Powers	Strategy and tactics in protein chemistry
John Cebra	The detailed structure of antibodies
William Clem	Biologic activities of antibodies other than specific ligand binding
John Cebra	Genetic control of the immune response
Dennis Barrett	Genetic control of early development: transcriptional
Dennis Barrett	Chromatin structure: facts and theories
Ru-chih Huang	Eukaryotic transcription: *in vitro* assay systems
Ru-chih Huang	Specific genes and gene products
Pien-chien Huang	Fine structure analysis of structural and regulatory genes by base sequencing
Pien-chien Huang	Sanger's approach to the anatomy of an RNA molecule
Pien-chien Huang	Review of special techniques relevant to sequence determination for DNA and RNA
Eric Weinberg	Cots, Rots, peaks and dots—an approach to studying gene organization and function
Eric Weinberg	Isolation and characterization of eukaryotic genes
T. Baldwin	Structural and functional aspects of bacterial luciferase
Dennis Powers	The structure, function and molecular ecology of fish hemoglobins
D. Yphantis	Equilibrium centrifugation
Gary Ackers	Physical techniques for studying protein-protein and protein-liquid interactions
Richard Rodewald	Transport of macromolecules across cell membranes
W. Kundig	Transport of small molecules across cell membranes

EXPERIMENTAL MARINE BOTANY

I. Consultant

Frank Loewus, State University of New York, Buffalo

II. Tutorial Committee

Ralph S. Quatrano, Oregon State University
Jerome A. Schiff, Brandeis University (Chairman)

III. Seminars

Joseph Ramus	Sulfation of *Porphyridium* cell surface polysaccharides
Samuel Beale	Chlorophyll biosynthesis
Scott Bingham	Sulfate utilization in *Prasiola stipitata*
Ami Ben-Amotz	Hydrogen evolution and photoreduction in algae
Anthony Liuzzi	Isotope procedures at MBL
Ralph Quatrano	*Fucus* embryogenesis
A. Trebst	Native and artificial energy conservation in photosynthetic electron flow
Mary Ann Henderson	Formic dehydrogenase in *Desulforvibrio gigos*
David Mauzerall	Light saturation in photosynthesis
James Saunders	Biosynthesis and localization of flavinoids and their enzymes
Frank Loewus	Ascorbic acid metabolism in plants
W. Stoeckenius	Bacteriorhodopsin
Martin Gibbs	C4 photosynthesis: current concepts
David Mauzerall	Essence of bacterial photosynthesis: reaction centers
Frank Loewus	Ascorbic acid metabolism in plants
Hans Gaffron	Sulfur dismutation in *Chlorobrium*
Jane Gibson	Nucleotide pools in growing photosynthetic bacteria
David Erbes	Nitrogen fixation enzymology

EXPERIMENTAL INVERTEBRATE ZOOLOGY

I. Consultants

F. A. Brown, Jr., Professor of Zoology, Northwestern University
C. Ladd Prosser, Professor of Physiology, University of Illinois
Alfred C. Redfield, Woods Hole Oceanographic Institution
W. D. Russell-Hunter, Professor of Zoology, Syracuse University
James Case, Professor of Biology, University of California, Santa Barbara

II. Instructors

Robert K. Josephson, University of California, Irvine, director of course
James Morin, University of California at Los Angeles, associate director of course
Carl J. Berg, Jr., City College, City University of New York
J. B. Jennings, University of Leeds, England
Ralph G. Johnson, University of Chicago
Ann E. Kammer, Kansas State University, Manhattan
Leonard Kirschner, Washington State University
S. K. Pierce, University of Maryland
Thomas J. M. Schopf, University of Chicago

III. Course Assistants

John C. Cornell, University of California, Berkeley, teaching assistant
Mary Cornell, course secretary
Douglas Fenner, University of Pennsylvania, teaching assistant

IV. Lectures

R. Josephson	Introduction to Woods Hole and to the course
S. K. Pierce	Sponges
R. Johnson	Environments of Cape Cod
R. Josephson	Introduction to coelenterates
R. Josephson	Coelenterates and the origin of nervous systems
S. Pierce	Molluscs
T. Schopf	Bryozoans
C. Berg	Annelids
A. Kammer	Arthropoda
J. Morin	Echinoderms, unique plumbers
R. Johnson	Plankton and dredging
J. Morin	Protochordates, etc.
T. Schopf	Origination and extinction of animal species
T. Schopf	Historical *vs.* equilibrium views of the evolution of life
J. Morin	Organization and coordination in colonial animals
S. Pierce	Diffusion, osmosis, salinity tolerance and volume regulation
S. Pierce	Molecular mechanisms of cell volume regulation with a fast look at anaerobiosis
J. Cornell	Ionic regulation and water permeability in spider crabs
L. Kirschner	Osmoregulation and active transport
F. A. Belamarich	Hemostasis in invertebrates
J. Jennings	Digestive physiology in invertebrates, especially acoelomates
R. Josephson	Relations between structure and function in striated muscle
R. Josephson	The design of very fast muscles
F. Lang	Crustacean neuromuscular systems
J. Morin	Bioluminescence
A. Kammer	Generation of rhythmic motor patterns
A. Kammer	Regulation of body temperature by invertebrates
C. Berg	Development of behavior of marine gastropods
C. Berg	Importance of invertebrates to behavioral theory

MARINE ECOLOGY

I. Consultants

Harlyn O. Halvorson, Professor of Biology, Brandeis University
J. Woodland Hastings, Professor of Microbiology, Harvard University
Lawrence B. Slobodkin, Professor of Biology, State University of New York
Roger Y. Stanier, Professor of Microbiology, Institut Pasteur, Paris
Edward O. Wilson, Professor of Biology, Harvard University

II. Instructors

Holger W. Jannasch, Senior Scientist, Woods Hole Oceanographic Institution, director of course
A. Jane Gibson, Associate Professor of Biochemistry, Cornell University
Robert E. Hungate, Professor of Microbiology, University of California, Davis
Edward R. Leadbetter, Professor of Microbiology, Amherst College

Kenneth H. Nealson, Assistant Professor, University of California, San Diego
Anatol Eberhard, Associate Professor, Ithaca College

III. Research Associates

Alex Keynan, Professor of Microbiology, The Hebrew University, Jerusalem
Carolyn Eberhard, Lecturer in Genetics, Cornell University
Craig D. Taylor, Assistant Scientist, Woods Hole Oceanographic Institution
Clark B. Interlied, Postdoctoral Fellow, University of Massachusetts, Amherst
Charles C. Remsen, Associate Scientist, Woods Hole Oceanographic Institution

IV. Laboratory Assistant

Mary A. van Holde-Ackerson, Marine Biological Laboratory

V. Special Lecturers

Mary M. Allen, Wellesley College
Ananda M. Chakrabarty, General Electric Research Laboratory
Arnold L. Demain, Massachusetts Institute of Technology
Joe C. Goldman, Woods Hole Oceanographic Institution
Robert L. Guillard, Woods Hole Oceanographic Institution
Harlyn O. Halvorson, Brandeis University
J. Woodland Hastings, Harvard University
Galen E. Jones, University of New Hampshire
Jerome J. Perry, North Carolina State University, Raleigh
John H. Ryther, Woods Hole Oceanographic Institution
Michael J. Wolin, University of Illinois, Urbana

VI. Lectures

H. W. Jannasch	Introduction to microbial ecology
	Principles of marine microbiology
	Theory and practice of the chemostat
	Applications of the chemostat in microbial ecology
	Experiments in deep-sea microbiology
R. E. Hungate	Comparative microbiology of digestion, I and II
	Life without air
	The rumen ecosystem, I and II
A. J. Gibson	Introduction to the photosynthetic bacteria
	The acquisition of nutrients by microorganisms
	The biology of *Bdellovibrio*
E. R. Leadbetter	Enrichment cultures
	How to make a living aerobically, I and II
	Growth and morphogenesis of *Sporocytophaga*
	Microorganisms of the tooth surface
K.H. Nealson	Bacterial genetics, I and II
	Symbiotic luminescent bacteria
A. Eberhard	Chemistry and regulation of bacterial bioluminescence
C. Eberhard	Bacteriophages in the environment
M. M. Allen	The ecology of blue-green bacteria
A. M. Chakrabarty	Genetic engineering in the clean-up of oil spills
A. L. Demain	The excretion of metabolites by microorganisms
J. C. Goldman	Continuous culture of photosynthetic organisms

R. L. GUILLARD	Influence of low nutrient concentrations on growth of marine phytoplankton
H. O. HALVORSON	The making and the breaking of dormancy in *Bacillus*
J. W. HASTINGS	Bioluminescence: a survey of functions and mechanisms
G. E. JONES	The elemental composition of marine microorganisms The microbial sulfur cycle in Oyster Pond
J. J. PERRY	Hydrocarbons as substrates and non-substrates for microorganisms Commensalistic growth of microorganisms in a model system involving hydrocarbons
C. C. Remsen	Ultrastructural survey of marine bacteria
J. H. RYTHER	Aquaculture
M. J. WOLIN	Species interaction in anaerobic systems
C. B. INDERLIED	Microbial evolution and ecology

NEUROBIOLOGY

I. INSTRUCTORS

MICHAEL V. L. BENNETT, Professor of Anatomy, Albert Einstein College of Medicine, co-director of course
JOHN E. DOWLING, Professor of Biology, Harvard University, co-director of course
ANTHONY L. F. GORMAN, Professor of Physics, Boston University School of Medicine
RODOLFO R. LLINAS, Professor of Physiology and Biophysics, University of Iowa
GEORGE PAPPAS, Professor of Anatomy, Albert Einstein College of Medicine
VICTOR P. WHITTAKER, Max-Planck Institute of Biophysical Chemistry, West Germany

II. LECTURES

GEORGE PAPPAS	Fine structure of the neuron: general consideration
GEORGE PAPPAS	The structure of chemically transmitting synapses in relation to function
GEORGE PAPPAS	The structure of electrotonic synapses in relation to function
STEVEN WAXMAN	The morphological specialization of the axon in relation to function
JOHN DOWLING	Anatomical analysis of the vertebrate retina
JACK ROSENBLUTH	The contractile apparatus and the myoneural junctions in some invertebrate non-striated muscle
RODOLFO LLINAS	The synaptic organization of the cerebellum
HERBERT LEVITAN	Physico-chemical basis of membrane potentials
HERBERT LEVITAN	The resting membrane potential of excitable cells: comparison with physico-chemical theory
HERBERT LEVITAN	Electrogenic cation pump: characteristics and contribution to membrane potential
HERBERT LEVITAN	Biophysical basis for membrane selectivity for ions
HERBERT LEVITAN	Drugs affecting the resting membrane potential: biophysical basis of action
HERBERT LEVITAN	Excitability and the nature of nerve action potentials
ICHIJI TASAKI	Bi-ionic action potentials and membrane macromolecules
JEFFREY BARKER	Pacemakers: introduction to mechanisms and regulation
JOHN DOWLING	Organization of visual systems: introduction to the *Limulus* lateral eye

George Wald	Visual pigments
John Dowling	Photoreceptor physiology
L. Frischkoff	Transduction in hair cells
John Dowling	The processing of visual information
N. Daw	Neurophysiology of color vision
M. Goldstein	The sensory cortex and behavior
R. Chappell	The median ocellus of the dragonfly—anatomy, physiology, and pharmacology
Rodolfo Llinas	General properties of chemically mediated synaptic transmission
Rodolfo Llinas	Depolarization-release coupling
P. W. Gage	Function of post synaptic membrane
M. V. L. Bennett	Physiology of electric organs
M. V. L. Bennett	Electroreception
W. T. Clusin	The skate electroreceptor
Rodolfo Llinas	Inhibition
M. V. L. Bennett	Electrical synapses
V. P. Whittaker	Biochemistry of the synapse
V. P. Whittaker	Subcellular fractionation techniques applied to nervous tissue
M. J. Dowdall	Transmitter synthesis and storage in the cholinergic and adrenergic systems: parallels and contrasts
V. P. Whittaker	Dynamics of vesicle formation and discharge
L. A. Barker	Biochemical pharmacology of the synapse
E. J. Simon	Biochemistry of receptors including the opiate receptor
E. A. Dravitz	Synaptic chemistry of the lobster nervous system
D. Soifer	Cyclic nucleotides in neuronal function
D. Soifer	Microtubules and microtubular protein in nervous system
M. V. L. Bennett	Interpretation of intracellularly recorded responses
M. V. L. Bennett	Control of simple effector systems
L. B. Cohen	Optical measurement of activity in a vertebrate central nervous system
M. V. L. Bennett	Teleological analysis of chemical and electrical transmission
R. Llinas	Electrophysiology of neuronal circuits in the cerebellar cortex
P. Witkovsky	Organization of the mesencephalic nucleus of selachians
C. Nicholson	Analysis of field potentials in the CNS
R. Llinas	Responses of the cerebellum to physiological stimuli

DEVELOPMENTAL BIOLOGY (January Course, 1974)

I. Instructors

Louis E. DeLanney, Ithaca College, coordinating instructor
Lester G. Barth, Marine Biological Laboratory
Lucena J. Barth, Marine Biological Laboratory
Annette W. Coleman, Brown University
John R. Coleman, Brown University
James D. Ebert, Carnegie Institution of Washington (Baltimore) and Marine Biological Laboratory
Joanne E. Fortune, Cornell University
David Walters, Harvard University

II. SPECIAL LECTURERS

ROBERT D. ALLEN, State University of New York at Albany
JOHN M. ARNOLD, University of Hawaii
FRANCIS P. BOWLES, Boston University Marine Program
SUSAN GERBI, Brown University
RICHARD GOSS, Brown University
JUDITH GRASSLE, Marine Biological Laboratory
ARTHUR G. HUMES, Boston University Marine Program
EDWARD F. MACNICHOL, JR., Marine Biological Laboratory
RUDOLPH SHELTEMA, Woods Hole Oceanographic Institution
DAVID C. SHEPHARD, Woods Hole
RAYMOND E. STEPHENS, Brandeis University
ALBERT SZENT-GYÖRGYI, Marine Biological Laboratory

III. LECTURES AND SEMINARS

JAMES D. EBERT	MBL—yesterday, today, tomorrow
JAMES D. EBERT	Interacting systems in development
JAMES D. EBERT	Fertilization—gamete physiology
ROBERT D. ALLEN	How amoebae move
JAMES D. EBERT	Fertilization; special cells for continuity
LOUIS E. DELANNEY	Oogenesis
FRANCIS P. BOWLES	Lobsters and lobstermen
LOUIS E. DELANNEY	Oogenesis, spermatogenesis
LOUIS E. DELANNEY	Relevance of genetic material
LESTER G. BARTH	Differentiation and development as seen in sponges and coelomates
ALBERT SZENT-GYÖRGYI	Electronic aspects of biology
LESTER G. BARTH	Differentiation and development as seen in spiralians
JOHN M. ARNOLD	Cytokinesis and development of cephalopoda
LESTER G. BARTH	Differentiation and development as seen in ascidians
ARTHUR G. HUMES	Post-embryonic development in copepoda
DAVID WALTERS	Differentiation and development as seen in arthropods
SUSAN GERBI	Chromosome structure
JAMES D. EBERT	Intracellular contact and cell regulation
JOHN R. COLEMAN	Modes of genetic analysis in development
JAMES D. EBERT	Congenital defects
JAMES D. EBERT	Continuity of genetic endowment: mitosis, cytokinesis
JUDITH GRASSLE	Population genetics of marine opportunistic species
LUCENA J. BARTH	Preorganogenetic development in animals
RAYMOND E. STEPHENS	Microtubules
ANNETTE W. COLEMAN	Models of plant development
RICHARD GOSS	Regeneration
JOHN R. COLEMAN	Models of tissue differentiation
JOHN R. COLEMAN	Reassessment of molecular and genetic mechanisms in development
RUDOLPH SHELTEMA	Ecology of marine benthic larvae
JOANNE E. FORTUNE	Hormonal control of gene action and development
DAVID C. SHEPHARD	Cytoplasmic inheritance
LOUIS E. DELANNEY	External influences on development
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Thomas, William A., Princeton University
Tsang, Monica Lik-Shing, Brandeis University
Vickers, Gail R., Max Planck Institute for Biophysical Chemistry, West Germany
Wallace, Richard W., Wayne State University
Watts, John A., University of Maryland
Westerfield, Monte, Duke University
Westly, Stephen K., Tulane University
Whitlatch, Robert B., University of Chicago
Yantorno, Robert E., University of Pennsylvania
Yulo, Teresa S., University of Rochester Medical School
Zakevicius, Jane, New York University School of Medicine
Zweig, Ronald, University of Oregon

Library Readers, 1974

Aaronson, Robert Peter, Assistant Professor, Mt. Sinai Medical School and The Rockefeller University
Adelberg, Edward A., Professor of Human Genetics, Yale University
Allen, Garland E., Associate Professor of Biology, Washington University
Armstrong, Philip B., Professor of Anatomy, College of Medicine, State University of New York, Upstate Medical Center
Bruns, Romaine R., Research Associate, Harvard University
Carlson, Francis D., Professor of Biophysics, The Johns Hopkins University
Child, Frank M., Professor of Biology and Acting Chairman of the Department, Trinity College
Clark, Arnold M., Professor of Biological Sciences, University of Delaware
Clifford, Sister Adele, Professor of Biology, College of Mount St. Joseph on the Ohio
Cobb, Jewel Plummer, Professor of Zoology, Dean of the College, Connecticut College
Coleman, Bernard D., Senior Fellow and Professor of Mathematics, Carnegie-Mellon University
Colwin, Laura Hunter, Adjunct Professor, School of Marine and Atmospheric Sciences, University of Miami
Copeland, Donald Eugene, Professor of Biology, Tulane University
Cornwall, M. Carter, Postdoctoral Research Associate, Vanderbilt University
Coward, Stuart J., Associate Professor, University of Georgia

DeHaan, Robert L., Professor of Anatomy and Physiology, Emory University
Driscoll, Egbert G., Professor of Geology, Wayne State University
Dunham, Philip B., Professor of Physiology, Syracuse University
Eder, Howard A., Professor of Medicine, Albert Einstein College of Medicine
Fussel, Catharine P., Assistant Professor of Biology, The Pennsylvania State University
Gabriel, Mordecai L., Dean, School of Science, Brooklyn College, The City University of New York
Gelfant, Seymour, Professor of Dermatology and Cell and Molecular Biology, Medical College of Georgia
German, James L., Senior Investigator and Director, Laboratory of Human Genetics, The New York Blood Center and Cornell University Medical College
Goudsmit, Esther Marianne, Assistant Professor, Department of Biological Sciences, Oakland University
Grant, Philip, Professor of Biology, University of Oregon
Green, James W., Professor of Physiology, Rutgers—The State University
Grundfest, Harry, Emeritus Professor and Special Lecturer in Neurology, Columbia University
Hillman, Peter, Associate Professor of Biophysics, Hebrew University of Jerusalem, Israel
Inoue, Sadayuki, Research Associate, McGill University, Canada
Isenberg, Irvin, Professor of Biophysics, Oregon State University
Isselbacher, Kurt J., Mallinckrodt Professor of Medicine, Harvard Medical School and Chief, Gastrointestinal Unit, Massachusetts General Hospital
Karush, Fred, Professor of Microbiology, University of Pennsylvania
Katz, Elton P., Professor, University of Connecticut Health Center
Keeferstein, Sister Mary Vincentia, Assistant Professor of Biology, Clarke College
Kelly, Robert E., Associate Professor, University of Illinois College of Medicine
Kempton, Rudolf T., Professor Emeritus of Biology, Vassar College
Kirschenbaum, Donald M., Associate Professor, College of Medicine, State University of New York, Downstate Medical Center
Korn, Edward D., Chief, Cellular Biochemistry and Ultrastructure Section, National Heart and Lung Institute, National Institutes of Health
Kosower, Edward M., Professor of Chemistry, Tel-Aviv University, Israel
Laderman, Aimlee, Instructor, School of Theoretical and Applied Science, Ramapo College of New Jersey
Le Douarin, Georges, Full Professor, Université de Nantes, France
Le Douarin, Nicole, Full Professor, Université de Nantes, France
Leighton, Joseph, Professor and Chairman, Department of Pathology, Medical College of Pennsylvania
Lochhead, John H., Professor of Zoology, University of Vermont
Marsland, Douglas A., Research Professor Emeritus, New York University
Mizell, Merle, Professor of Biology, Tulane University
Palmer, John D., Professor of Biology, New York University
Patterson, Paul H., Assistant Professor, Harvard Medical School
Plocke, Donald J., Chairman, Department of Biology, Boston College
Ratliff, Floyd, Professor, The Rockefeller University
Renninger, George H., Associate Professor of Physics, University of Guelph, Canada
Rowland, Lewis P., Professor and Chairman, Department of Neurology, Columbia University
Rubinow, Sol I., Professor of Biomathematics, Cornell University Medical College
Sager, Ruth, Professor, Hunter College—The City University of New York
Saunders, John, W., Jr., Professor of Biological Sciences, State University of New York at Albany
Schlesinger, R. Walter, Professor and Chairman, Department of Microbiology, Rutgers Univestity Medical School, College of Medicine and Dentistry of New Jersey
Shaw, Evelyn, Senior Research Associate, Stanford University
Shedlovsky, Theodore, Professor, The Rockefeller University
Shemin, David, Professor of Biochemistry, Northwestern University
Sherman, Irwin W., Professor of Zoology, University of California, Riverside
Sonnenblick, B. P., Professor of Zoology, Rutgers—The State University
Strittmatter, Philipp, Professor of Biochemistry, University of Connecticut Health Center
Terebey, Nicholas, Assistant Professor, New York University College of Dentistry

Tweedell, Kenyon S., Professor of Biology, University of Notre Dame
Van Holde, K. E., Professor of Biophysics, Oregon State University
Wainio, Walter, Professor of Biochemistry, Rutgers—The State University
Webb, H. Marguerite, Chairman, Professor of Biological Sciences, Goucher College
Weiss, Leon, Professor of Anatomy, The Johns Hopkins University School of Medicine
Wheeler, George E., Professor of Biology, Brooklyn College—The City University of New York
Wilson, Thomas Hastings, Professor of Physiology, Harvard Medical School
Wittenberg, Beatrice A., Assistant Professor of Physiology, Albert Einstein College of Medicine
Wolken, Jerome J., University Professor of Biophysics, Carnegie-Mellon University
Yntema, Chester L., Professor of Anatomy, State University of New York, Upstate Medical Center
Zacks, Sumner I., Professor of Pathology, University of Pennsylvania, School of Medicine and Pennsylvania Hospital

Students, 1974

All students listed completed the formal course program. Asterisk indicates completing post-course research program.

EMBRYOLOGY

Anderson, David M.
Barnett, Thomas R.
Crain, William R., Jr.
Dolecki, Gregory J.
Duncan, Roger
Dworkin, Mark B.
Gelfand, Robert A.
Giorno, Ralph C.
Goldberg, Robert B.
Harpold, Michael M.
Higashinakagawa, Toru, T. H.
Higgins, Ratchford C.
Holland, Christie A.
Kleene, Kenneth C.
Kovacic, Roger T.
Lecanidou, Rena
Moore, Gordon P.
Olexa, Stephanie A.
Ruderman, Joan V.
Sklar, Joseph H.
Sun, Tung-Tien
Uziel, Mayo

PHYSIOLOGY

*Abruzzini, Anthony F.
Anderson, James M.
Bergtrom, Gerald
*Bloom, Joseph W.
Bockus, Beverly J.
*Clark, Darryl E.
*Cohen, Charles M.
Collins, Tucker
Ditzian, Ruth
*Dunlap, Jay C.
Evans, John A.
*Fallon, Justin R.
*Forbes, Douglass J.
Gottlieb, Avrum I.
*Greaney, George S.
Greve, Jeffrey M.
*Griffith, Linda M.
Haimo, Leah T.
Harris, H. William, Jr.
Katula, Karen S.
*Kipps, Thomas J.
Kriegler, Michael P.
Kunkle, Herman M., Jr.
*Lanpher, Gregory B.
Lasky, Laurence A.
*Liepins, Andrejs
McClellan, Deborah A.
*McCue, Robert O.
Mickiewicz, Cristina W.
*Nagle, David P., Jr.
O'Connor, Clare M.
Rich, Steven A.
Shaw, Phillip H.
Silberstein, Laura H.
Smith, Malcolm A.
Strausbaugh, Linda D.
Townsend, Janis K.
*Wey, Ghanglin A.
*Williams, Keith D.
*Woodrum, Diane T.

EXPERIMENTAL MARINE BOTANY

Campisi, Judith
Dokow, Shoshana
Geiger, Adam
Hellerstein, Marc
Kaye, Jeffrey A.
LaZerte, Bruce
Lindroth, Kenneth J.
Lowy, Howard W.
Piccioni, Richard G.
Relosky, Patricia M.
Walker, Henry A.

INVERTEBRATE ZOOLOGY

Ardell, Jeffery L.
Bucklin, Ann C.
Cederblad, Ann V.
Culp, Patricia A.
Detmers, Patricia A.
Gallagher, Jane C.
Garner, Judy A.
Hill, John E.
Jacob, William F.
Korper, Susan H.
Kritzer, Gordon L.
Kroeker, John P.
Lasfargues, John E.
Leonard, Janet L.
McBride, Margaret M.
Miller, Molly B. F.
Moore, Margaret
Myers, Joseph B., Sr.
Phelps, Harriette L.
Porter, Mary E.
Rajasekhara, Koosappa
Reed, Charlene
Rhoderick, John C.
Romano, Frank A., III
Samaras, George M.
Shorey, Jeannette M.
Socci, Robin R.
Wiltse, Wendy I.
Wood, Lois A.

MARINE ECOLOGY

Binkowski, Gloria J.
*Cerniglia, Carl E.
Colberg, Patricia J.
*Fontaine, James W.
Haumann, Don
Haygood, Margo J.
*Healy, Joseph B.
*Hoffman, Jacqueline L.
Karl, David M.
Ozretich, Robert J.
*Singer, Howard J.
*Slowinski, Eugene J.
*Smith, Charles J.
*Thomas, Kathleen S.

NEUROBIOLOGY

Ballou, Edmund
Epstein, Kerry
Harris, Andrew
Hauger, Steven
Honig, Marcia
Langford, George
Lohmann, Suzanne
Martinez, Joseph, Jr.
Newkirk, Robert
Schairer, John
Wong, Fulton
Zottoli, Steven

FRONTIERS IN RESEARCH AND TEACHING PROGRAM

Langford, George
Martinez, Joseph, Jr.
Myers, Joseph B.
Newkirk, Robert
Phelps, Harriette L.
Rajasekhara, Koosappa

3. Fellowships and Scholarships, 1974

Bio Club Scholarship:

Robin Socci

Gary H. Calkins Scholarship:

Pat A. Detmers
Margaret McBride

Lucretia Crocker Scholarship:

Judith Campisi
Don Haumann
Joseph B. Healy

4. Training Programs

Fertilization and Gamete Physiology Research Training Program

I. Instructors

Charles B. Metz, University of Miami, program director
Robin A. Wallace, Oak Ridge National Laboratory, co-director
John Chamberlain, University of Michigan
Michael Edidin, The Johns Hopkins University
David Epel, University of California, San Diego
S. S. Koide, The Population Council
David M. Phillips, The Population Council
Joel Rosenbaum, Yale University
Sheldon S. Segal, The Population Council

II. Assistants

Kay Ellen Selman, Harvard University, EM assistant
Ruth Edidin, program secretary
Margot Balboni, photographic assistant

III. Trainees

Susan Atlas, University of Maryland
Mary Lee Sparling Barber, California State University
Lester I. Binder, Yale University
Bonnie S. Dunbar, University of Miami
William R. Eckberg, Michigan State University
James D. Johnson, Veterans Hospital, Atlanta
Diane K. Juricek, Emory University
Suzanne Jackowski, University of Tennessee
Thomas J. Sardner, University of Illinois
Utpalendu S. Maitra, Columbia University, College of Physicians and Surgeons
Carl S. Parker, Washington University
George W. Smith, Union College
Joseph L. Turner, University of Colorado
Doris A. Wall, Cornell University
Eileen D. Hickey Weber, Russell Sage College
Carol A. M. Ziomek, The Johns Hopkins University
Barry R. Zirkin, The Johns Hopkins University
David R. Bishop, Harvard University
Franklin D. Collins, University of California, San Diego

IV. Lectures

C. R. Austin	Capacitation in mammals
R. Steinhardt	Ionic mechanisms in the activation of eggs
J. Chamberlain	Histones and hybrids
Stephen Roth	Cell surface glycosyl transferase enzymes
Elias Lazarides	Actin fiber structure in non-muscle cells as revealed by immune fluorescent antibody techniques
John Bongaarts	A demographic analysis of human reproduction
David Phillips	Studies on sperm structure and motility
Paul Demeny	The world demographic situation: trends and prospects

TONY BELLVE	Basic chromosomal proteins during spermatogenesis
GEOFFREY MCNICOLL	The economics of population policy
MARTIN GOROVSKY	DNA and histone of genetically active and inactive nuclei

EXCITABLE MEMBRANE PHYSIOLOGY AND BIOPHYSICS TRAINING PROGRAM

I. CONSULTANTS

W. J. ADELMAN, JR., National Institute of Neurological Diseases and Stroke
K. S. COLE, National Institute of Neurological Diseases and Stroke
J. W. MOORE, Duke University Medical Center
L. J. MULLINS, University of Maryland, School of Medicine

II. INSTRUCTORS

D. E. GOLDMAN, Medical College of Pennsylvania, program director
K. S. COLE, National Institute of Neurological Diseases and Stroke
J. W. MOORE, Duke University Medical Center
D. GILBERT, National Institute of Neurological Diseases and Stroke
L. J. MULLINS, University of Maryland, School of Medicine
L. J. DEFELICE, Emory University
G. EHRENSTEIN, National Institute of Neurological Diseases and Stroke
R. J. FRENCH, National Institute of Neurological Diseases and Stroke
D. JM. POUSSART, Université Laval, Canada
W. N. ROSS, Yale University
G. STRICHARTZ, Yale University
R. VILLEGAS, IVIC, Caracas, Venezuela
W. K. CHANDLER, Yale University School of Medicine
W. J. ADELMAN, JR., National Institute of Neurological Diseases and Stroke
T. NARAHASHI, Duke University Medical Center
M. M. DEWEY, State University of New York at Stony Brook
W. K. BLASIE, University of Pennsylvania
H. LECAR, National Institute of Neurological Diseases and Stroke
L. E. MOORE, Case Western Reserve University
C. L. PROSSER, University of Illinois
C. M. ARMSTRONG, University of Rochester
J. Y. LETTVIN, Massachusetts Institute of Technology

III. ASSISTANTS

P. GREIF, Haverford College
J. STOCKLER, Drexel University

IV. TRAINEES

P. G. P. ANG, University of California, Berkeley
C. J. DEUTSCH, University of Pennsylvania
G. DROOGMANS, University of Leuven, Belgium
H. S. HALIN, Massachusetts Institute of Technology
C. S. HIN, University of Miami
R. L. NOVACK, University of Pennsylvania
L. L. ODETTE, University of Toronto, Canada
F. SIEBERT, University of Freiburg
R. P. SWENSON, Washington State University
P. P. VAN BOGAERT, University of Antwerp, Belgium
S. YOUNG, University of California, San Diego

V. LECTURES

D. GILBERT	The biology and ecology of the squid
D. E. GOLDMAN	Physical and electrochemistry of membrane transport, I

D. E. Goldman	Physical and electrochemistry of membrane transport, II
K. S. Cole	The electrical properties of membranes
K. S. Cole	The strategy of the voltage clamp
J. W. Moore	Operational amplifiers
J. W. Moore	Preamplifiers
J. W. Moore	Voltage clamp circuits
J. W. Moore	Cable theory
G. Strichartz	The chemical composition of excitable membranes
M. M. Dewey	Electron microscopy of excitable membranes
L. J. Mullins	Passive transport of ions
L. J. Mullins	Active transport of ions
W. K. Chandler	Voltage clamp of the squid axon
W. K. Chandler	The Hodgkin-Huxley formulation
W. K. Chandler	Univalent ions and selectivity
W. J. Adelman	Ion competition in channels
W. J. Adelman	The periaxonal space
C. L. Armstrong	Sodium channels and gating currents
C. L. Armstrong	Potassium channels
D. E. Goldman	Divalent cations
D. E. Goldman	Surface charges
L. E. Moore	Myclinated nerve
W. K. Blasie	X-Ray diffraction of membranes
W. K. Chandler	Striated muscle, I
W. K. Chandler	Striated muscle, II
T. Narahashi	Drugs and toxins, I
T. Narahashi	Drugs and toxins, II
J. W. Moore	The mode of action of TTX
C. L. Prosser	The electrophysiology of smooth muscle
H. Lecar	Channels and carriers in lipid bilayers
G. Ehrenstein	Excitability in bilayers
H. Lecar	Channel noise in excitable membranes
J. Y. Lettvin	A transistor model for excitability
D. E. Goldman	Excitability models: I
D. E. Goldman	Excitability model: II
D. E. Goldman	Excitability models: III
L. DeFelice	Electrical noise from heart cell membranes in culture

Research Program in Experimental Marine Botany

I. Senior Investigators

Martin Gibbs, Brandeis University
Frank A. Loewus, State University of New York at Buffalo
Ralph S. Quatrano, Oregon State University
Jerome A. Schiff, Brandeis University
Joseph S. Ramus, Yale University

II. Associate Investigators

Hans Gaffron
David B. Dickinson, University of Illinois, Champaign-Urbana

III. Junior Investigators

Ami Ben-Amotz
Samuel Beale
Scott Bingham
David Erbes
Judith Heady

MARY ANN HENDERSON
ELIOT HERMAN
KENNETH L. HOWARD
STEVEN A. JOHNSON
JAMES SAUNDERS

RESEARCH PROGRAM IN HISTORY OF BIOLOGY

I. SENIOR INVESTIGATORS

FREDERICK B. CHURCHILL, Indiana University
WILLIAM COLEMAN, The Johns Hopkins University
CAMILLE LIMOGES, Université de Montréal, Canada
MARY P. WINSOR, University of Toronto, Canada

5. TABULAR VIEW OF ATTENDANCE, 1970–1974

	1970	*1971*	*1972*	*1973*	*1974*
INVESTIGATORS—TOTAL	532	554	561	523	508
Independent	324	322	328	312	302
Library Reader	73	76	76	86	75
Research Assistants	135	156	157	125	131
STUDENTS—TOTAL	142	130	119	123	146
Invertebrate Zoology	41	29	38	32	30
Embryology	28	28	19	20	21
Physiology	31	33	31	41	40
Experimental Botany	19	22	14	15	11
Ecology	23	18	17	15	14
Developmental Biology					30
TRAINEES—TOTAL	33	44	46	50	53
TOTAL ATTENDANCE	707	728	726	696	707
Less persons represented in two categories	0	0	1	0	0
	707	728	725	696	707
INSTITUTIONS REPRESENTED—TOTAL	191	219	210	239	222
FOREIGN INSTITUTIONS REPRESENTED	21	27	25	40	31

6. INSTITUTIONS REPRESENTED, 1974

Albert Einstein College of Medicine
American Museum of Natural History
Amherst College
Atlanta University
Barbara-Scotia College
Barnard College
Boston College
Boston University
Boston University School of Medicine
Brandeis University
Brooklyn College, The City University of New York
Brown University
Browne and Nichols
California Institute of Technology
California, University of, Berkeley
California, University of, Davis
California, University of, Irvine
California, University of, Los Angeles
California, University of, Riverside
California, University of, San Diego
California, University of, Santa Barbara
California State University
California State University, Northridge
Carnegie Institution of Washington
Carnegie-Mellon University
Case Western Reserve University
Case Western Reserve University, Medical School
Chicago, University of

Cincinnati, University of
Cincinnati, University of,
College of Medicine
City College, The City University
of New York
Clarke College
College of Mount St. Joseph on the Ohio
Colorado College
Columbia University
Columbia University, College of
Physicians and Surgeons
Connecticut, University of
Connecticut, University of, Health Center
Connecticut, University of, Medical School
Connecticut College
Cornell University
Cornell University Medical College
Dartmouth College
Delaware, University of
Delaware State College
Denver, Univeristy of
De Pauw University
Drew University
Duke University
Duke University Medical Center
Emory University
Federal City College
Florida, University of
Florida State University
George Mason University
Georgetown University
Georgia, University of
Goucher College
Hampshire College
Harvard Medical School
Harvard University
Haverford College
Hawaii, University of
Herbert Lehman College, The City
University of New York
Houston, University of
Howard University
Hunter College, The City University
of New York
Illinois, University of
Illinois, University of,
College of Medicine
Illinois, University of, Urbana-Champaign
Illinois Institute of Technology
Indiana University
Institute of Muscle Research
Iowa, University of
Iowa State University
Ithaca College
Jersey City State College
Johns Hopkins University, The
Johns Hopkins University, The,
School of Hygiene
Johns Hopkins University, The,
School of Medicine
Juniata College
Kansas State University
Kentucky, University of
Kirkland College
Kresge Eye Institute
Long Island University
Louisiana State University
Lowell Technological Institute
Marine Research Foundation, Inc.
Marquette University
Maryland, University of
Maryland, University of, School of Medicine
Massachusetts, University of
Massachusetts General Hospital
Massachusetts Institute of Technology
Medical College of Georgia
Medical College of Ohio at Toledo
Medical College of Pennsylvania
Meharry Medical College
Mellon Institute of the Carnegie-Mellon
University
Miami, University of
Miami, University of,
School of Marine and Atmos. Science
Miami, University of,
School of Medicine
Miami University
Michigan, University of
Minnesota, University of
Mt. Sinai School of Medicine,
The City University of New York
National Heart and Lung Institute
National Institute of Arthritis,
Metabolism & Digestive Diseases
National Institute of Mental Health
National Institute of Neurological
Diseases and Stroke
National Institutes of Health
New College
New York Blood Center, The
New York State Department of Health
New York University
New York University, College of Dentistry
New York University Medical Center
New York University, School of Medicine
New York Zoological Society
North Carolina, University of
North Carolina, University of, at Chapel Hill
North Carolina State University, Raleigh
North Dakota State University
Northwestern University
Notre Dame, University of
Oak Ridge National Laboratory
Oakland University
Oberlin College
Oregon, University of
Oregon State University

Pennsylvania, University of
Pennsylvania, University of, School of Medicine
Pennsylvania Hospital
Pennsylvania State University
Pittsburgh, University of
Pomona College
Population Council, The
Princeton University
Purdue University
Ramapo College of New Jersey
Rhode Island, University of
Rochester, University of
Rochester, University of, Medical School
Rockefeller University, The
Rollins College
Rush Medical College
Rutgers—The State University
Rutgers University Medical School
Scripps Institution of Oceanography
Seton Hill College
Smith College
Stanford University
State University of New York
State University of New York, Downstate Medical Center
State University of New York, Upstate Medical Center
State University of New York at Albany
State University of New York at Binghamton
State University of New York at Buffalo
State University of New York at Stony Brook
Syracuse University
Temple University
Tennessee, University of
Texas Christian University
Texas, University of
Texas, University of, Arlington
Texas, University of, Austin
Texas, University of, at Houston
Texas, University of, Medical Branch
Trinity College
Tulane University
Vanderbilt University
Vassar College
Vermont, University of
Veterans Administration Hospital, Brooklyn
Virginia, University of
Virginia State College
Washington & Jefferson College
Washington, University of
Washington State University
Washington University
Washington University, School of Medicine
Wayne State University
Wellesley College
Wesleyan University
West Florida, University of
Wisconsin, University of
Woods Hole Oceanographic Institution
Yale University
Yale University School of Medicine

FOREIGN INSTITUTIONS REPRESENTED, 1974

Athens, University of, Greece
American University of Beirut, Lebanon
Antwerp, University of, Belgium
Basel, The University of, Switzerland
Chile, University of, Chile
College of Science & Technology, Nigeria
Dalhousie University, Canada
Glasgow University, Scotland
Guelph, University of, Canada
Hebrew University, The, Israel
Institut für Biologie III der Universität Tübingen, Germany
International Institute of Genetics and Biophysics, Italy
Istanbul, University of, Turkey
Kyoto, University of, Japan
Laboratory of Cell Biology, Italy
Leeds, University of, England
Los Andes University Medical School, Venezuela
Marine Biological Association, England
Max Planck Institute for Biophysical Chemistry, West Germany
McGill University, Canada
Middlesex Hospital Medical School, England
Montreal, University of, Canada
Münster, University of, Germany
Nantes, Université de, France
New South Wales, University of, Australia
Ottawa, University of, Canada
Paris, University of, France
Stockholm University, Sweden
Tel-Aviv University, Israel
Toronto, University of, Canada
Université Laval, Canada

7. FRIDAY EVENING LECTURES, 1974

June 28

DANIEL BRANTON . . . . Freeze-etch studies of membrane organization
Harvard University

July 5	
JOEL ROSENBAUM Yale University	Assembly of microtubules *in vitro*
July 12	
KENNETH H. MANN Dalhousie University	Seaweeds, sea urchins and lobsters: some ecological interactions in the coastal zone
July 18	
CARL GUSTAF BERNHARD Royal Swedish Academy of Sciences Alexander Forbes Lecturer at the Marine Biological Laboratory	Electrophysiological analysis of the prenatal development of recipient functions in the mammalian cerebral cortex
July 19	
CARL GUSTAF BERNHARD	The compound eye in light and darkness—a human physiologist's adventures with the insect eye
July 26	
JOEL E. BROWN Vanderbilt University	An illuminating eye of *Limulus*
August 2	
ROBERT L. DEHAAN Emory University	Functional differentiation of the embryonic heart-cell membrane
August 9	
K. O. EMERY Woods Hole Oceanographic Institution	Oceanography and offshore oil production
August 16	
MICHAEL EDIDIN The Johns Hopkins University	Histocompatibility genes and cell physiology
August 23	
GERALD WEISSMANN New York University School of medicine	Metchnikoff revisited: phagocytosis and inflammation

8. MEMBERS OF THE CORPORATION, 1974

Including Action of 1974 Annual Meeting

Life Members

ADOLPH, DR. EDWARD F., University of Rochester School of Medicine and Dentistry, Rochester, New York 14627

BEAM, DR. HAROLD W., Department of Zoology, State University of Iowa, Iowa City, Iowa 52240

Behre, Dr. Elinor M., Black Mountain, North Carolina 28711
Bertholf, Dr. Lloyd M., 1228 Gettysburg Dive, Bloomington, Illinois 61701
Bodansky, Dr. Oscar, Department of Biochemistry, Memorial Cancer Center, 444 East 68 Street, New York, New York 10021
Bradley, Dr. Harold C., 2639 Durant Avenue, Berkeley, California 94704
Brown, Dr. Dugald E. S., Cape Haze, Box 426, Placida, Florida 33946
Burdick, Dr. C. Lalor, The Lalor Foundation, 4400 Lancaster Pike, Wilmington, Delaware 19805
Clarke, Dr. George L., 44 Juniper Road, Belmont, Massachusetts 02178
Cole, Dr. Elbert C., 2 Chipman Park, Middlebury, Vermont 05753
Cowdry, Dr. E. V., 4580 Scott Avenue, St. Louis, Missouri 63110
Diller, Dr. Irene C., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038
Diller, Dr. William F., 2417 Fairhill Avenue, Glenside, Pennsylvania 19038
Ferguson, Dr. James K. W., 56 Clarkshaven St., Thornhill, Ontario, Canada
Fischer, Dr. Ernst, 3110 Manor Drive, Richmond, Virginia 23230
Furth, Dr. Jacob, 99 Fort Washington Ave., New York, New York 10032
Gaffron, Dr. Hans, Department of Biology, Institute of Molecular Biophysics, Conradi Building, Florida State University, Tallahassee, Florida 32306
Galtsoff, Dr. Paul, National Marine Fisheries Service, Woods Hole, Massachusetts 02543
Gray, Dr. Irving E., Department of Zoology, Duke University, Durham, North Carolina 27701
Hamburger, Dr. Viktor, Department of Zoology, Washington University, St. Louis, Missouri 63110
Hess, Dr. Walter, 787 Maple Street, Spartanburg, South Carolina 29302
Hibbard, Dr. Hope, 366 Reamer Place, Oberlin, Ohio 44074
Hisaw, Dr. F. L., 5925 W. Plymouth Drive, Corvallis, Oregon 97330
Hoadley, Dr. Leigh, 985 Memorial Drive, Cambridge, Massachusetts 02138
Hollaender, Dr. Alexander, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830
Irving, Dr. Laurence, University of Alaska, College, Alaska 99701
Kaan, Dr. Helen, P. O. Box 665, Woods Hole, Massachusetts 02543
Kahler, Robert, Box 423, Woods Hole, Massachusetts 02543
Kille, Dr. Frank R., 340 Albany Shaker Road, Londonville, New York 12211
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V. REPORT OF THE LIBRARIAN

During the summer months the library received and catalogued over 1,400 books purchased by the Oceanographic Institution to expand our book coverage of ocean engineering, physics, geology and marine policy. Renovation of Room 306 (the equivalent of three labs in the Lillie Building) was completed and most of the new books are in this area. W.H.O.I. also added 20 new subscriptions to journals in the above subjects and in many cases purchased volumes back to 1964.

The Oceanographic staff completed the move to the new Quisset campus and this has brought about a greater effort on our part to get material to them as rapidly as possible. We deliver books and copies of articles twice daily and handle many more phone requests now that they're two miles from the library. They recently hired a Research Reference Librarian to oversee their part of the collection, specifically book acquisitions, and this should relieve the burden placed on our very small staff.

The National Marine Fisheries Service library here in Woods Hole has duplicated their card catalog and interfiled their cards with ours in the main catalog, thereby placing their collection within reach of all scientists using our library. Hopefully the few small collections at the Oceanographic, including the Documents Collection, will also be handled in the same manner.

Interlibrary loan requests continue to come in at a rate of 20 to 30 per day. Xerox billing in 1973 was $24,000; in 1974, it jumped to $40,000. Two staff members now work full time in the xerox department.

We added 99 new journal titles to the periodicals section and 3,800 bound volumes and books making a total of 155,564 volumes at the end of 1974.

VI. REPORT OF THE TREASURER

The market value of the General Endowment Fund and the Library Fund at December 31, 1974, amounted to $1,606,881.00 and the corresponding securities are entered in the books at a value of $1,642,174.00. This compares with values of $2,154,552 and $1,633,192 respectively, at the end of the preceding year. The average yield on the securities was 6.10% of the market value and 5.97% of the book value. Uninvested principal cash was $1,513.00. Classification of the securities held in the Endowment Fund appears in the Auditor's Summary of Investments.

The market value of the Pooled Securities at December 31, 1974, amounted to $918,181.00 as compared to book values of $1,100,195.00. These figures compare with values of $1,088,355.00 and $930,340.00 respectively, at the close of the preceding year. The average yield on the securities was 5.67% of the market value and 4.73% of the book value. Uninvested principal cash was in the amount of $933.00.

The proportionate interest in the Pool Fund Account of the various funds, as of December 31, 1974, is as follows:

Pension Funds	32.42%
General Laboratory Investment	26.80%
F. R. Lillie Memorial Fund	1.91%
Other:	
Bio Club Scholarship Fund	0.50%
Rev. Arsenius Boyer Scholarship Fund	0.60%
Garry N. Calkins Fund	0.57%
Allen R. Memhard Fund	0.11%
Lucretia Crocker Fund	2.07%
E. G. Conklin Fund	0.35%
Jewett Memorial Fund	0.25%
M. H. Jacobs Scholarship Fund	0.18%
Herbert W. Rand Fellowship	17.69%
Mellon Foundation	8.35%
Mary Rogick Fund	1.83%
Swope Foundation	4.60%
Osterhaut Fund	1.77%

Unrestricted gifts from foundations, societies and companies amounted to $53,044.00.

During the year we administered the following grants and contracts:

Investigators	*Training*	*MBL Institutional*
4 NIH	3 NIH	1 NSF
4 NSF	1 Waksman Ecology	
1 Damon Runyon Cancer		
1 Merrill Trust		
1 API		
1 EPA		
12	4	1

Most of the federally funded research grants provided for reimbursement of indirect costs, on a cost per square foot basis for the laboratory space assigned to each project. The provisional rate of $12.25 per square foot set by NIH for 1973 was finalized at $14.89, and a provisional rate of $17.20 was established for 1974. A maximum provisional rate of $12.25 per square foot for NSF grants remained in effect for 1974. Training courses supported by NIH grants were funded for indirect costs at a rate of 8% of allowable direct costs.

The following is a statement of the auditors:

To the Trustees of Marine Biological Laboratory, Woods Hole, Massachusetts:

We have examined the balance sheet of Marine Biological Laboratory as of December 31, 1974, and the related statements of operating expenditures and income and funds for the year then ended. Our examination was made in accordance with generally accepted auditing standards and, accordingly, included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances. We previously examined and reported on the 1973 financial statements.

In our opinion, the aforementioned financial statements (pages 74 to 78) present fairly the financial position of Marine Biological Laboratory at December 31, 1974 and 1973, and its operating expenditures and income for the years then ended, and the changes in its funds for the year ended December 31, 1974, in conformity with the accounting principles referred to in Note A to the financial statements applied on a consistent basis.

The supplementary schedule (page 79) included in this report was obtained from the Laboratory's records in the course of our examination and, in our opinion, is fairly stated in all material respects in relation to the financial statements taken as a whole.

Boston, Massachusetts
April 4, 1975

COOPERS & LYBRAND

It will be noted from the operating statement that the Laboratory activities for the year under review, amounted to a figure of a little over 2.1 million dollars, which amount is 285 thousand more than the preceding year.

ALEXANDER T. DAIGNAULT,
Treasurer

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1974 and 1973

Investments	*1974*	*1973*
Investments held by Trustee:		
Securities, at cost (approximate market quotation, 1974—$1,606,881; 1973—$2,154,552)	$ 1,642,174	$ 1,633,192
Cash	1,513	2,433
	1,643,687	1,635,625
Investments of other endowment and unrestricted funds:		
Pooled investments, at cost (approximate market quotation, 1974—$918,181; 1973—$1,088,355)	1,100,195	930,340
Other investments, at cost (approximate market quotation, 1974—$1,114,636; 1973—$1,122,526 in 1973)	1,451,508	1,366,230
Cash	933	1,571
Due from current fund	63,490	189,168
	$ 4,259,813	$ 4,122,934

Plant Assets		
Land, buildings, library and equipment, at cost	12,454,336	12,454,336
Less allowance for depreciation (Note A)	3,007,343	2,726,028
	$ 9,446,993	$ 9,728,308

Current Fund Assets		
Cash	56,060	65,310
Investments	311,437	—
Accounts receivable (U. S. Government, 1974—$39,300; 1973—$33,739)	269,087	252,589
Inventories of supplies and bulletins	53,841	48,533
Other assets	7,668	5,912
Due to endowment funds	(63,490)	(189,168)
	$ 634,603	$ 183,176

MARINE BIOLOGICAL LABORATORY

BALANCE SHEETS

December 31, 1974 and 1973

Invested Funds	*1974*	*1973*
Endowment funds given in trust for benefit of Marine Biological Laboratory	$ 1,643,687	$ 1,635,625
Endowment funds for awards and scholarships		
Principal	455,403	449,777
Unexpended income	69,927	65,715
	525,330	515,492
Unrestricted funds functioning as endowment	1,804,605	1,726,603
Retirement fund	398,392	357,439
Investments of other endowment and unrestricted funds—accumulated loss	(112,201)	(112,225)
	$ 4,259,813	$ 4,122,934

Plant Funds		
Funds expended for plant, less retirements	12,454,336	12,454,336
Less allowance for depreciation charged thereto	3,007,343	2,726,028
	$ 9,446,993	$ 9,728,308

Current Fund Liabilities and Balances		
Accounts payable and accrued expenses	29,245	20,677
Advance subscriptions	46,690	35,899
Unexpended grants—research	51,666	39,987
Unexpended balances of gifts for designated purposes	683,351	124,124
Current fund	(176,349)	(37,511)
	$ 634,603	$ 183,176

The accompanying note is an integral part of the financial statements.

A. *Accounting Principles*:

The following accounting principles have been reflected in the accompanying financial statements:

1. Investments are stated at cost.
2. Investment income is recorded on a cash basis.
3. Operating income is recorded when earned.
4. Expenses are recorded on an accrual basis.
5. Depreciation has been provided for plant assets at annual rates ranging from 1% to 5% of the original cost of the assets.

MARINE BIOLOGICAL LABORATORY

Statement of Operating Expenditures and Income for the years ended December 31, 1974 and 1973

	1974						
	Salaries and Wages	*Other Costs and Expenses*	*Depreciation (Note A)*	*Total*	*Charged to Grants*	*1974 Total*	*1973*
Operating expenditures:							
Instruction		$ 45,419	$ 75,584	$ 121,003	$241,222	$ 362,225	$ 345,947
Research		60,825	80,606	141,431	400,337	541,768	461,807
Dormitories	$ 23,412	68,564	101,251	193,227		193,227	176,754
Dining		115,873		115,873		115,873	103,376
Library	46,333	22,872	17,442	86,647		86,647	86,407
Back sets, serials and binding		79,622		79,622		79,622	71,915
Biological Bulletin	7,632	49,411		57,043		57,043	61,102
Support services:							
Apparatus	64,571	107,734		172,305		172,305	131,901
Supply	99,071	77,333	4,418	180,822		180,822	171,318
Administration	127,038	183,217		310,255		310,255	250,978
Plant operation	171,066	203,407	2,015	376,488		376,488	338,016
Grant expenditures for support services					15,000	15,000	15,810
Other		43,491		43,491		43,491	42,309
Total	$539,123	$1,057,768	$281,316	1,878,207	656,559	2,534,766	2,257,640

	Fees	*Other*	*Total*			
Income:						
Instruction	$109,673		$109,673	$241,222	$350,895	$337,232
Research	426,937		426,937	400,337	827,274	687,821
Dormitories		219,673	219,673		219,673	200,052
Dining		127,766	127,766		127,766	114,058
Library	30,293	49,223	79,516		79,516	70,270
Biological Bulletin		70,446	70,446		70,446	60,511
Support services:						
Apparatus		58,393	58,393		58,393	51,028
Supply		90,914	90,914		90,914	72,090
Administration		35,965	35,965		35,965	23,844
Investment income		182,402	182,402		182,402	160,435
Gifts used for current expense		53,044	53,044		53,044	39,217
Allowance for indirect costs		50,879	50,879		50,879	40,648
Grants for support services				15,000	15,000	15,810
Other		11,547	11,547		11,547	15,529
Total	$ 566,903	$950,252	1,517,155	$656,559	2,173,714	1,888,545
Excess of current expenditures and depreciation over current income			361,052		361,052	369,095
Reduction in plant funds for depreciation			281,316		281,316	281,975
Excess current expenditures			$ 79,736		$ 79,736	$ 87,120

The accompanying note is an integral part of the financial statements.

MARINE BIOLOGICAL LABORATORY

Statement of Funds

for the year ended December 31, 1974

	Balance December 31, 1973	*Gifts and Other Receipts*	*Invest-ment Income*	*Used for Current Expenses*	*Transfers*		*Other Ex-pendi-tures*	*Balance December 31, 1974*
					To	*From*		
Invested funds	$4,122,934	$ 81,413	$216,298	$182,402	$64,728		$43,158	$4,259,813
Unexpended grants—research	$ 39,987	722,674		710,995				$ 51,666
Unexpended balances of gifts for designated purposes	$ 124,124	613,486				$ 5,626	48,633	$ 683,351
Current fund	$ (37,511)	(79,736)				59,102		$ (176,349)
		$1,337,837	$216,298	$893,397	$64,728	$64,728	$91,791	
Other gifts and receipts		613,486						
Grants for research, training and support		722,674						
Appropriated from current income and other		81,413						
(1) Excess of current expenditures over income		(79,736)						
		$1,337,837						
Scholarship awards							14,589	
Payments to pensioners							18,107	
Loss on sale of securities							10,462	
Other							48,633	
							$91,791	

The accompanying note is an integral part of the financial statements.

MARNIE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1974

	Cost	*Per-cent of Total*	*Market Quotations*	*Per-cent of Total*	*Investment Income 1974*
Securities held by Trustee:					
General endowment fund:					
U. S. Government securities	$ 23,685	1.8	$ 23,818	1.8	$ 1,166
Corporate bonds	530,477	40.4	411,715	31.3	27,848
Preferred stocks	31,094	2.4	12,875	1.0	1,200
Common stocks	726,116	55.4	866,373	65.9	48,387
	1,311,372	100.0	1,314,781	100.0	78,601
General educational board endowment fund:					
Corporate bonds	126,590	38.3	85,882	29.4	7,927
Commerical paper	52,000	15.7	52,000	17.8	2,949
Common stocks	152,212	46.0	154,218	52.8	8,588
	330,802	100.0	292,100	100.0	19,464
Total securities held by Trustee	$1,642,174		$1,606,881		98,065
Investments of other endowment and unrestricted funds:					
Pooled investments:					
Corporate bonds	274,200	24.9	174,092	19.0	12,950
Commercial paper	213,000	19.4	213,000	23.2	16,428
Preferred stocks	62,207	5.6	49,413	5.4	2,695
Common stocks	550,788	50.1	481,676	52.4	19,982
	1,100,195	100.0	$ 918,181	100.0	52,055
Other investments:					
U. S. Government securities	27,764	1.9	27,476	2.5	1,645
Other bonds	15,029	1.1	10,575	.9	750
Common stocks	614,192	42.3	283,788	25.5	21,443
Preferred stocks	2,871	0.2	1,145	0.1	104
Real estate	17,550	1.2	17,550*	1.6	—
Short-term commercial notes	774,102	53.3	774,102	69.4	50,076
	1,451,508	100.0	$1,114,636	100.0	74,018
Total investments of endowment and other funds	$2,551,703				
Total					224,138
Custodian's fees charged thereto					(7,840)
Total investment income					$216,298

*At cost.

Reference: *Biol. Bull.*, **149**: 80–95. (August, 1975)

ELECTRICAL ACTIVITY ASSOCIATED WITH LUMINESCENCE AND OTHER COLONIAL BEHAVIOR IN THE PENNATULID *RENILLA KÖLLIKERI*

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Although great advances have recently been made in the understanding of some coelenterate nervous systems very little is known of conducting systems in the colonial anthozoans, the corals and pennatulids. This is in contrast to the situation in the intensively-studied solitary anthozoans where the conducting systems are well described. They possess a through-conducting, non-polarized nerve net responsible for both fast and slow muscle contractions (Pantin, 1935, Batham, Pantin and Robson, 1960; Robson and Josephson, 1969; McFarlane, 1969a, 1973a, b, 1974). In addition, there are two slow conduction systems, the SS1 and SS2 (McFarlane, 1969a) which are inhibitory in nature and are active during such complex behavior as the preparatory feeding response of *Tealia felina,* the shell climbing response in *Calliactis parasitica* and the spontaneous contractions of the column musculature in *Calliactis* (McFarlane, 1969b, 1970, 1973a, b; McFarlane and Lawn, 1972). In the latter case there is an interaction between the nerve net and the SS2.

The basic structure of sea anemones is similar to that of pennatulid and coral polyps but it is not known whether this similarity extends to the conduction systems. Furthermore, little is known about how individuals in the colonies interact. On the basis of behavioral studies Horridge (1957) proposed that a colonial nerve net coordinates protective withdrawal in coral colonies. Anatomical studies by Buisson (1970) indicate three separate nerve nets in the sea pen *Veretillum cynomorium,* suggesting that multiple colonial conduction systems might exist in colonial pennatulids. Further work is obviously necessary to confirm these suggestions. In hydrozoans, colonial conduction systems have been studied in *Obelia* (Morin and Cooke, 1971a, b), *Hydractinia* (Stokes, 1974), and *Proboscidactyla* (Spencer, 1974). In all of these there are indications that the pathways for the colonial systems are epithelial.

The pennatulid, *Renilla,* has been extensively studied but without regard to its electrophysiology. Interest has focused principally on its luminescence which has been studied from biochemical (Cormier, Hori, Karkhanis, Anderson, Wampler, Morin and Hastings, 1973; Cormier, Hori and Anderson, 1974), behavioral (Parker, 1920; Buck, 1953, 1955, 1973; Nicol, 1955a, b) and ultrastructural (Spurlock and Cormier, 1975) viewpoints. Luminescence in *Renilla* is produced by a luciferin-luciferase reaction generating blue light (488 nm) which serves to excite an associated fluorescent moiety with resultant emission of the green light (508 nm) characteristic of the living animal. Of importance to our present study

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is the fact that luminescence in this and some other coelenterates is light-inhibited with the site of inhibition being associated with either the neuroeffector junction or the light triggering process (Nicol, 1955a; Davenport and Nicol, 1955; Kreiss and Cormier, 1967).

Luminescence appears in the intact colony as waves of light traversing the upper surface of the rachis in a non-decremental, facilitating manner. Nicol (1955b) proposed that the luminescent waves reflect activity in a colonial nerve net. Parker (1920) studied four colonial activities in *Renilla köllikeri* and proposed that three of these, luminescence, polyp withdrawal and rachidial contraction, are controlled by the same nerve net, and that the fourth, peristalsis, is under separate control through some pacemaker system.

This well-described array of colonial and individual zooid activities offers useful indices of the integrative processes occuring within the *Renilla* colony. These, together with the convenient size and morphology of the colony, prompted the following electrophysiological studies directed towards gaining further insight into the means by which individual and colonial behavior interact in an anthozoan colony.

Materials and Methods

Good descriptions of the morphology of *Renilla köllikeri* are given by Parker (1919, 1920), Waterman (1950), Buck (1955, 1973) and Lyke (1965). Salient features of the gross morphology of *Renilla* appear in Figure 1.

Specimens of *Renilla köllikeri* were collected locally by divers and maintained in the laboratory in fresh or recirculating sea water (10–20° C). The light regime to which they were exposed was irregular and undetermined, but approximated 12 hours light, 12 hours dark. Since they were never observed to feed on any of the standard food sources such as *Artemia* larvae, they were not fed during the period of maintenance. (During the course of unrelated experiments on *Renilla* it was found that, while *Artemia* nauplii are immobilized by nematocysts upon contacting *Renilla* tentacles, feeding does not follow. The same polyps fed avidly upon small bits of frozen and thawed beef liver or fresh *Mytilus* muscle.) This starvation did not seem to have adverse effects and the colonies could be kept in good condition in the laboratory for up to 8 weeks.

Electrical recordings were made with suction electrodes similar to those of Josephson (1966). Tip diameters ranged from 0.2–0.4 mm. Signals were amplified with a Grass P511R A.C. amplifier (time constant 3 msec) and displayed on a Tektronix 434 storage oscilloscope. While the electrodes could be attached to all parts of the colony, the numerous spicules that cover the rachis prevented secure attachment and consequently reduced signal strength. The polyps, being free from spicules, provided good recording sites. Stimuli (5–15 volts, 0.1 msec) from a Grass S4 stimulator were applied through a suction electrode.

Luminescence was recorded through a tapered glass light guide projecting onto the photocathode of an EMI Type 9781 B photomultipler tube with the cathode operated at 935 volts from a Fluke 409A DC power supply. The photomultiplier anode was connected directly to the oscilloscope. The records of the luminescence indicate relative light intensity. Photometric recordings and visual observations of luminescence were supplemented by observations with an image

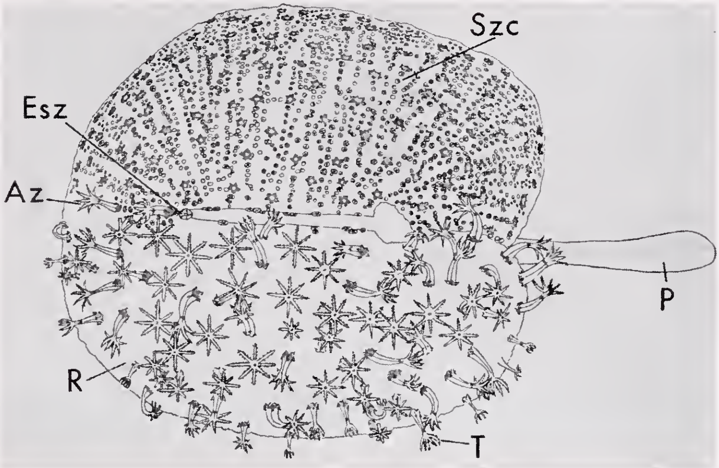

FIGURE 1. Diagrammatic representation of *Renilla köllikeri* as viewed from above. The upper half of the diagram represents a colony in which the zooids are withdrawn, the lower half one in which the autozooids (Az) are expanded. The siphonozooid clusters (Scz) have been omitted from the lower half for clarity; × 1. Az indicates autozooid; Esz, large exhalent siphonozooid; P, peduncle; R, rachis; Scz, siphonozooid cluster; and T, tentacle of autozooid showing pinnules.

intensifier (Night Vision Sight, Varo Inc.) with amplification of approximately 14,000 ×.

Inhibition of luminescence by ambient illumination was avoided by using red light during the experiments. Once the colonies had been dark adapted for about

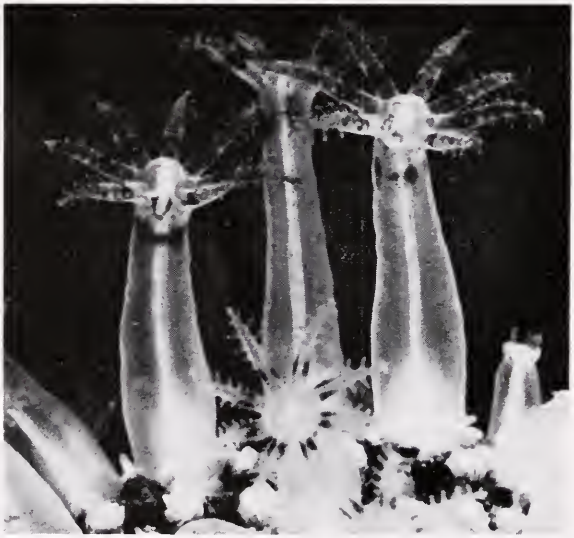

FIGURE 2. A photograph showing some of the fine detail of the polyps of *Renilla köllikeri;* × 5.5.

40 minutes, red light sufficient for necessary manipulation of the recording apparatus did not seem to affect luminescence.

RESULTS

Electrical activity

An electrical stimulus applied to any part of the colony produces two through-conducted impulses. Because their conduction delays are almost identical, they appear as a complex multipolar impulse (Fig. 3a). The two components of this complex are, however, separable on the basis of refractory periods and stimulus thresholds. Stimuli that are just above threshold produce a single pulse (Fig. 3b) that is conducted throughout the colony in an all-or-nothing manner. This is basically a negative-going monophasic pulse of 3–4 μV mean amplitude and 30 msec duration. Both the amplitude and duration usually increase slightly at the beginning of a series of these pulses (Fig. 3c) and diminish towards the end. The shape also varies with the recording site and duration of electrode attachment. In the rachis it is conducted at a rate of 6–7 cm/sec (16° C) with a relative refractory period of 250 msec. Conduction up the column of the autozoids is faster, 10–20 cm/sec (16° C); but, due to difficulties in measuring the resting length of the polyps, figures for the conduction rate in this area cannot be given more accurately. For reasons given in the discussion, these pulses are termed nerve net (NN) pulses.

When the stimulating voltage is increased, the recorded impulse is complicated by the triggering of the second pulse. On rare occasions when the stimulating electrode has been attached for an hour or so, the threshold for the second pulse is lower than that for the NN and it can be evoked alone. In all other instances and for all stimulus durations the threshold for the NN is lower. This second pulse is called the Biphasic (Bp) pulse, a term derived from its shape. The initial deflection is positive and it has a duration of 40 msec, a mean peak to peak amplitude of 7 μV (Fig. 3) and a relative refractory period of 450 msec.

If a train of stimuli is applied to the colony, the delay between the stimulus artifact and the Bp pulse increases while that before the NN pulse does not. This leads to a degree of separation between the pulses and aids in identification. In addition, adequate mechanical stimuli produce both NN and Bp pulses, but since they do not appear on the recordings simultaneously as do electrically stimulated pulses, they can be identified easily and clearly (Fig. 3d). Both NN and Bp pulses can be recorded from all parts of the colony although, for reasons mentioned earlier, the best records were obtained from the polyps. Neither pulse can be recorded in the presence of isotonic $MgCl_2$ solution.

The electrical basis of colonial activities

Although it is clear from the above observations that there are two separate colonial conduction systems it has not as yet been possible to identify either a pathway or a function for the Bp system. In contrast, the NN system can be shown to be the conduction system responsible for controlling three interconnected colonial activities.

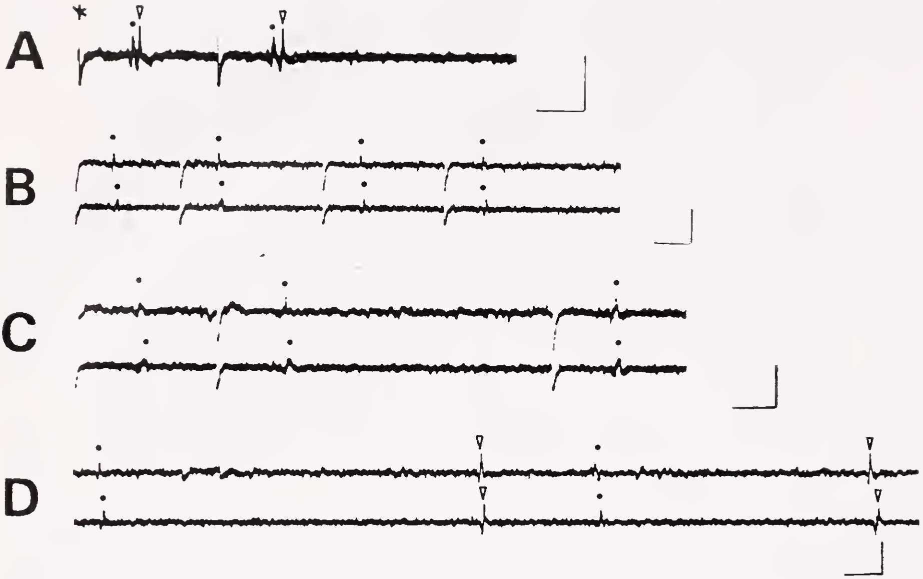

FIGURE 3. (A) Electrical activity recorded through a single electrode attached to the column of an autozooid. Each of two distantly applied electrical stimuli of approximately 12 V produces two pulses. The artifact produced by the first stimulus is marked with a star. In this and subsequent records; ordinate represents 5 μV; abscissa, 200 msec; closed circle. Nerve Net (NN) pulse; open triangle, Biphasic (Bp) pulse. (B) Electrical activity recorded simultaneously from two separate autozooids. The stimuli were of lower intensity, approximately 6 V, than in (A) and only NN pulses were recorded. (C) NN pulses recorded in the same manner as those in (B). However, it is evident that both the amplitude and duration of the NN pulses increase during the series. (D) Electrical activity recorded simultaneously from two separate autozooids in response to a single mechanical stimulus applied to a distant part of the rachis. Note that while a single electrical stimulus of sufficient intensity will only produce single NN and Bp pulses (Figure 3A) a single mechanical produces several.

Luminescence. Observations of previous workers (Agassiz, 1850; Parker, 1920; Buck, 1953, 1955, 1973; Nicol, 1955a, b) were largely confirmed. In response to a train of electrical stimuli or a single mechanical stimulus waves of light travel across the rachis away from the point of stimulation. Two or three stimuli are required to affect luminescence which then summates with additional stimuli and may then eventually fatigue (Nicol, 1955a, b). In this manner the system resembles a classic coelenterate neuro-effector system.

The luminescent tissue is confined to the siphonozooids and to the calices, column and crown of the autozooids, confirming Buck (1973). Electrical recordings made during luminescent activity indicate that the nerve net is responsible for the control and propagation of luminescence. In these studies the light guide was placed to collect the light emitted by the polyp to which the recording electrode was attached. This arrangement was best achieved using the large exhalent siphonozooid (Fig. 1, Esz), since this polyp does not present the problems associated with the mobility of the highly contractile autozooids. Although this arrangement allows the electrical and luminescent activity of a single polyp to be recorded simultaneously it does not totally exclude the luminescent contributions of neighboring polyps. This contribution is usually minimal but when the luminescence is bright, as occurs after extensive facilitation, the recorded luminescence may not accurately reflect that emitted by the polyp under study (Fig. 4b).

The recordings indicate that an NN pulse precedes the onset of luminescence (Fig. 4a). As before, each electrical stimulus elicits an NN pulse. After the second pulse, luminescence appears and following the third, the intensity increases. When both NN and Bp pulses are stimulated, it can be seen that luminescence follows only the NN pulse (Fig. 4b, c). On one occasion when only Bp pulses were stimulated, no luminescence was produced (Fig. 4d).

Accurate measurements of the delay between the NN pulse and the onset of luminescence have proved difficult. This is partly because the luminescent tissue viewed by the light guide is not a single unit but a multiplicity of separate luminescing sites which are not activated simultaneously, and partly because the light emitted by neighboring polyps could not be totally excluded. It can be said, however, that the delay is small and in the range 20–50 msec. This is in keeping with the fact that the rate at which luminescence travels across the rachis (Parker, 1920; Nicol, 1955a, b; Buck, 1973) differs little from the conduction rates of the NN pulses.

So far as can be ascertained, there is no difference between the electrical activity that can be recorded from dark adapted colonies and that which can be recorded from colonies that have been illuminated with bright light so as to inhibit their luminescence.

Recordings made during "frenzy" (Buck, 1973) (Fig. 4e) indicate that the spontaneous, erratic waves of light that characterize this behavior are the result of spontaneous activity in the nerve net. The recordings are indistinct because the stimuli required to induce "frenzy" are considerable and seem to cause almost total fatigue of the recorded potentials (see discussion). In all cases, however, small NN pulses could be observed.

Contrary to the findings of Buck (1973), no seasonal variation in the luminescent capabilities of autozooid crowns was observed. It was apparent, however, that the luminescent tissue in this area can respond to NN pulses independently from that in the siphonozooids. Frequently these two areas respond to stimulation together but it is not uncommon to find that the siphonozooids luminesce first and may take part in several waves of luminescence before the autozooids begin to respond. On occasion the autozooid crowns may not respond at all even though the siphonozooids luminesce normally. At other times the first few stimuli may cause luminescence in the autozooid crowns and this can be seen to travel over the colony as a wave of luminescence elevated above the rachis (J. G. Morin, personal communication, confirmed by authors). Siphonozooids do not necessarily luminesce at the same time and may not do so until luminescence in the crowns has fatigued and diminished.

Polyp withdrawal. The mechanism by which *Renilla* autozooids withdraw has been described by Parker (1920). He found that when stimulated sufficiently, individual autozooids can be made to withdraw without affecting their neighbors. However, general withdrawal of autozooids throughout the colony could only be

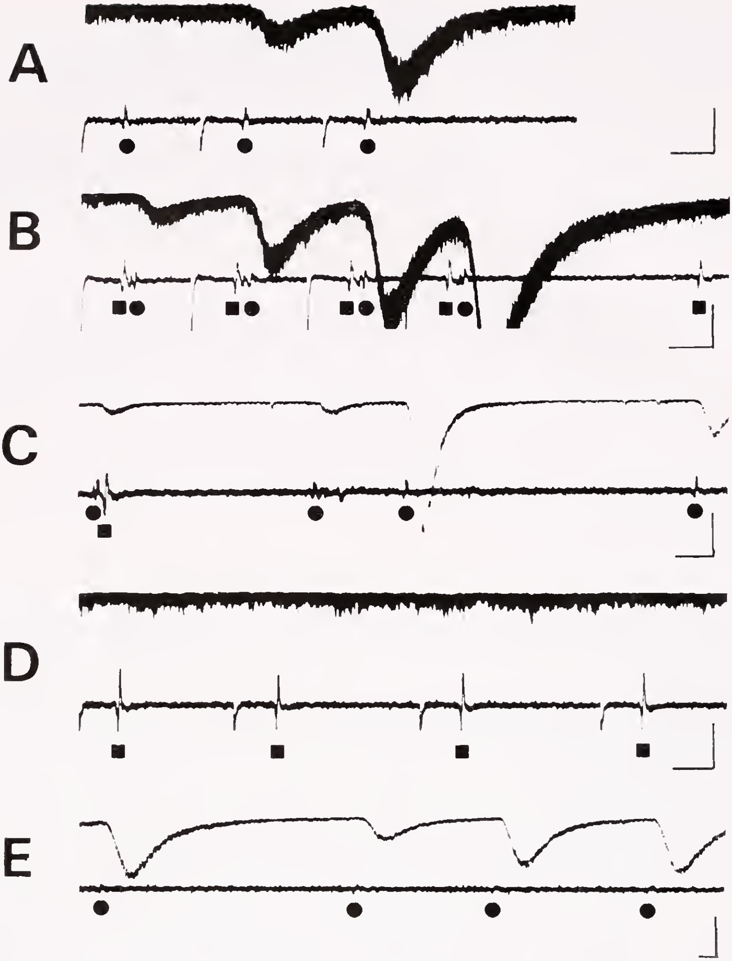

FIGURE 4. Simultaneous displays of electrical (lower traces) and luminescent activity (upper traces) recorded from the exhalent siphonozooid in the manner described in the text. (A) Stimuli of low intensity (6 V) produce only NN pulses. Luminescence appears after the second NN pulse and increases in intensity following the third. (B) Stronger stimuli (12 V) produce both NN and Bp pulses. Luminescence is recorded immediately after NN pulses but not after Bp pulses. Towards the end of the stimulus series the NN pulses and onset of luminescence fall out of step for reasons discussed in the text. A spontaneous or unstimulated Bp appears at the end of this record and is not associated with luminescence. Note that the fourth stimulus artifact is obscured by a peak on the luminescence record. (C) Luminescence and electrical activity recorded after a single mechanical stimulus was applied to the rachis. The stimulus leads to a series of NN pulses and one Bp pulse with luminescence being recorded only after the NN pulses. (D) Recordings of both electrical and luminescent activity made on an occasion when it was possible to stimulate only Bp pulses. No luminescence was recorded. (E) Recordings made during "frenzy". The NN pulses recorded are small for reasons discussed in the text. Note that each burst of luminescence is preceded by such a pulse. In this figure Bp pulses are marked by closed squares.

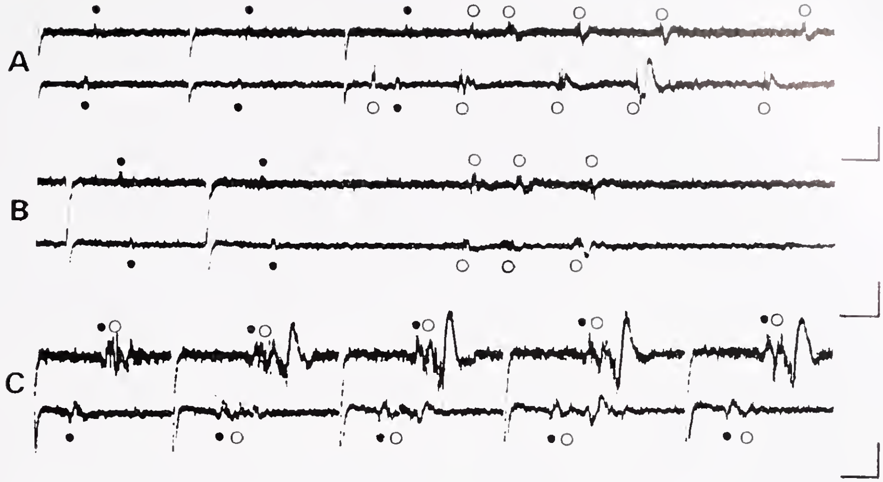

FIGURE 5. Electrical activity associated with autozooid withdrawal. (A) Recordings from two separate electrodes attached to separate autozooids. These records illustrate the events following the fourth of six electrical stimuli applied to a distant part of the colony. Each stimulus produces an NN pulse. After the sixth stimulus ZNN pulses (open circles) and associated muscle action potentials are recorded. (B) Electrical activity recorded through two electrodes attached at different levels on the same autozooid. The upper trace is recorded from the base of the column and the lower trace is recorded from the region just below the crown. Note that whereas the NN pulses travel up the column, the ZNN pulses travel down. (C) Electrical activity recorded as in (A). Here the ZNN pulses occur 1:1 with NN pulses. See text for details.

produced by severe stimulation of a polyp, although if the stimuli were applied to the rachis or peduncle, it could be elicited with less difficulty.

General polyp withdrawal can be effected by a series of stimuli (mechanical or electrical). There does not, however, seem to be any regularity in the sequences or patterns of autozooid withdrawal following such stimuli; some polyps withdraw completely, others partially, and still others not at all. In addition, the manner by which a colony responds to a particular degree of stimulation can differ from day to day.

When an autozooid polyp does withdraw because of electrical or mechanical stimulation it does so in a step-wise, sequential manner. First, there is a symmetrical contraction of the pinnules on the tentacles. This may occur after a single electrical stimulus but more commonly a series of at least three is required. Once the pinnules have contracted, each additional stimulus produces a further response. The first is a twitch of the tentacles followed by a symmetrical contraction of those same tentacles and later of the oral disk. At this stage the polyp may begin to withdraw into its cavity in the rachis regardless of whether additional stimuli are given, although a further stimulus is often necessary. Once the polyp has begun to withdraw, no further stimuli are necessary and the autozooid will disappear into the rachis by bending and contracting its column in a jerky manner. Although siphonozooids do contract, all the observations of polyp withdrawal were made on autozooids. This is because autozooid withdrawal is clear and distinctive and because recordings can be made concurrently with observations.

Stimulation of the colonial nerve net is sufficient to initiate general polyp withdrawal. These stimuli produce NN pulses which can be recorded from the column of autozooids (Fig 5a). After a series of stimulated NN pulses have been recorded a burst of multiphasic pulses is observed (Fig. 5a, b). Simultaneous recordings from adjacent polyps indicate that these pulses can only be recorded from the polyp in which they originate. Simultaneous recordings from two electrodes attached at different levels to the same polyp indicate that while NN pulses are conducted up the column of the autozooids these multiphasic pulses are always conducted downwards (Fig. 5b). The appearance of these pulses coincides with the onset of polyp withdrawal after NN activity. In addition these pulses can be recorded just after an electrode has been attached to a polyp with sufficient force to make it withdraw. It would therefore seem that they are associated with polyp withdrawal.

These multiphasic pulses are composed of two units. The first (indicated by open circles in Fig. 5a, b) is a small, short duration (4 μV, 30 msec) monophasic pulse of invariant shape in any one preparation. It is immediately followed by a second unit that increases in size and duration early in a series and then diminishes towards the end. The sequence is best seen in Fig. 5a.

It is conceivable that the second unit is a movement artifact caused by the contraction of the column but since its temporal relationship to the first unit is the same regardless of the position of the recording electrode, it is more likely that it represents a muscle action potential produced and facilitated by activity in some conduction system operating within the autozooids. Because of its invariant shape, the first unit is considered to be a direct recording of that activity. As will be shown in the discussion, the pathway for this activity is probably neuronal. Consequently, the first unit will be termed the Zooid Nerve Net pulse (ZNN pulse) with the second being referred to as the associated muscle action potential.

Similar recordings have been made from the mesenteries of the sea anemone *Metridium* (Robson and Josephson, 1969) and from the hydroid *Tubularia* (Josephson, 1974). In both these cases, a small invariant pulse immediately preceeds a larger variable one; the latter being interpreted as a muscle action potential, the former as activity in the conduction system activating that muscle. Furthermore, in both these cases, it is believed that the conduction system involved is a nerve net.

In certain preparations the electrical activity associated with withdrawal differs from that described above. The ZNN pulses and the muscle action potentials are recorded as before but, instead of appearing as a burst following a series of NN pulses, they occur singly each time an NN pulse reaches the polyp (Fig. 5c). They travel down the column as before and the polyp twitches and partially withdraws as each ZNN pulse is produced. The reason for this difference in behavior is unclear.

Parker (1920, page 507) noted that "the stimulus to zooid contraction is always productive of phosphorescence". A similar relationship was observed here. When a series of stimuli is applied to a dark adapted colony, luminescence appears after two or three stimuli and increases in intensity with subsequent ones. As noted earler the crowns of the autozooids do not necessarily luminesce at the same time as the siphonozooids and in many cases do not respond at all.

However, an autozooid will not withdraw without the crown of that autozooid luminescing.

Rachidial contraction. The third colonial behavior that Parker (1920) suggested as controlled by his one nerve net is rachidial contraction. This behavior can best be described as a symmetrical, slow contraction of muscles in the rachis which converts the colony from the usual flat shape to a domed or curled one. This contraction is produced by NN pulses. Stimulation of any part of the colony produces the contraction, the number and frequency of the stimuli dictating the size of the contraction. The most extensive contractions require prolonged stimulation and this is usually sufficient to induce both luminescence and polyp withdrawal.

Peristalsis. Peristalsis is characterized by more or less rhythmically generated slow, spontaneous waves of contraction passing over the rachis and peduncle. The waves originate at the point where the rachis and peduncle join, and proceed around the periphery of the rachis as a symmetrical pair progressing at a rate of about 1 to 2 mm/sec to meet at the point on the rachis opposite to the point of origin of the peduncle. This phenomenon was well described by Parker (1920) who suggested that these waves of contraction are controlled by some pacemaker system situated in the area where the rachis and peduncle join. No electrical activity could be recorded in association with peristalsis. Furthermore, no pulses conducted at a rate similar to that at which peristalsis travels were observed. Two somewhat unusual observations may, however, throw some light on the situation. On one occasion, the peristaltic waves were seen to originate at the margin of the rachis furthest from the peduncle and to pass over the rachis towards the usual point of origin, as waves of reverse peristalis. On another instance the waves originated at the usual site but, rather than traveling over the rachis as a pair of waves, crossed the rachis alternately, first on one side then the other. The concept of a pacemaker situated where the peduncle and rachis meet cannot adequately explain the first observation while the second might indicate that the two elements of the wave are separate, although usually simultaneously activated.

Discussion

Colonial organisms are characterized by effectors regularly distributed among the individuals that comprise the colony. Individuals may operate either independently or, under certain conditions, as elements of a colonial, or supra-organismal unit. In order for this latter behavior to occur there must be some means by which similar effectors are linked, as possibly through a colonial conduction system. Such a colonial conduction system could operate in two ways. It could either directly control the several effectors or else do so indirectly by activating some intermediate pathways, for example, a conducting system exclusive to the individual, which in turn controls the effectors. In *Renilla* we find two colonial conduction systems; of these, one, the NN system, operates in both of the ways suggested.

The NN system is responsible for colonial integration. Pulses associated with activity in this conduction system are recorded from all parts of the colony. The fact that the amplitude and duration of the recorded pulses increases (Fig. 3c) and later decreases during a stimulus series suggests that they might be potentials recorded from epithelial or myoepithelial cells as a result of activity in some underlying conduction system. In sea anemones, similar pulses have been interpreted in this manner and since they reflect activity in an underlying nerve net have been termed nerve net pulses (Josephson, 1966; McFarlane, 1969a). The morphological basis of the conduction system responsible for propagating these pulses in *Renilla* has not been determined. However, because the recorded pulses resemble muscle action potentials and since propogation is blocked by $MgCl_2$ it would appear that the recorded pulses are muscle action potentials, or potentials from epithelial cells (Ball and Case, 1973), that are stimulated and facilitated by activity in an underlying nerve net. Since these pulses are interpreted as reflecting nerve net activity they have been termed nerve net (NN) pulses. If this is the case, the pathway in this organism differs from that in the colonial hydroids mentioned earlier in being neuronal rather than epithelial.

It is interesting to find that a single stimulus produces one NN pulse that can be recorded from all parts of the colony. This finding would appear to negate in *Renilla* the necessity for the use of models such as those of Horridge (1957) and Josephson, Reiss and Worthy (1961) to explain incremental spread of impulses through a nerve net. The apparent absence of the need for interneural facilitation is contrary to the findings of Nicol (1955a). Using incisions to restrict the pathway of luminescent waves across the rachis he found that in such preparations there were failures in the conduction of some of the waves across the tissue bridges which could be explained, at least in part, by a requirement for interneural facilitation made apparent by the narrow bridges. The reason for the difference between these two findings is uncertain. NN pulses can be recorded from *Renilla* in the absence of stimulation at frequencies which, although low, may serve to keep the colony in a facilitated state. Incisions such as those made by Nicol may interfere with the spontaneous pulses and expose a requirement for interneural facilitation.

On the other hand a mechanism described by Josephson *et al.* (1961) might be present. If there are only a limited number of junctions requiring facilitation in an otherwise through-conducting nerve net, the general appearance of the colony would be one of through-conduction. If, however, the pathway is restricted, the small proportion of facilitating junctions may have an influence on the manner of conduction and produce facilitating transmission.

The colonial NN interacts directly with two effector systems. First, luminescent tissues in the siphonozooids and in the bases of the autozooids are stimulated by NN pulses. Luminescence is recordable after the second or third of a series of NN pulses and increases in intensity with subsequent pulses (Fig. 4a, b). This system therefore resembles a classic coelenterate neuroeffector system and strengthens the argument that NN pulses reflect nerve net activity. This in itself is not conclusive evidence, however, since epithelial effector junctions could display similar facilitation.

Flashing frequency is limited by the refractory period of the luminescent system itself rather than that of the NN. Nicol (1955b) found the relative refractory period of the luminescent system to be 500 msec. Measurements of the relative refractory period of the NN show that the figure is smaller, 250 msec. A similar situation to this occurs in jellyfish where the frequency of bell pulsations is limited by the refractory period of the muscles in the bell rather than by the nerves supplying them (Bullock, 1943; Passano, 1965).

The second effector system with direct NN control governs rachidial contraction. Recently this response has been shown to be an element of behavior by which *Renilla* escapes from the predatory nudibranch, *Armina* (Kastendiek, 1975). The size of the rachidial contraction is dependent on the number and frequency of the NN pulses, in the manner of the situation in sea anemones where slow muscle contractions of the column musculature are controlled by nerve net pulses, with contraction size depending on the number and frequency of those pulses (McFarlane, 1974).

The polyp withdrawal response is an example of indirect control of colonial effectors by the NN. Contraction of the autozooids is associated with activity in a conduction system within the polyps, the ZNN. A well developed nerve net has been observed in the mesenteries of *Renilla* (P. A. V. Anderson, in preparation). In sea anemones the muscles on the mesenteries and the nerves supplying them are responsible for the withdrawal of the anemones (Josephson, 1966; Robson and Josephson, 1969). Since similarly located morphological units are present in *Renilla* and since withdrawal involves column contraction, as in sea anemones, it would seem likely that in *Renilla* the mesenteric nerves and muscles are active during polyp withdrawal, and that the potentials recorded during withdrawal, the ZNN pulses, are conducted in these same nerves. Hence we suggested the use of the term Zooid Nerve Net pulses for this activity.

This direct innervation by the ZNN of the muscles responsible for withdrawal has been utilized by the colony to coordinate withdrawal of its component individuals. Rather than have dual control of these muscles through the colonially conducted NN pulses and the ZNN system, the NN system functions, in this instance, by activating the ZNN to produce the required withdrawal. Consequently, activity in the NN would be expected to initiate activity in the ZNN in all autozooids causing their simultaneous contraction. This does occur occasionally, but more often there is much variation in the relationship between the NN pulses and the ZNN pulses they induce. A similar situation is found in the hydroid *Obelia geniculata* (Morin and Cooke, 1971b) in which colonially conducted luminescence pulses (LP's) drive the contraction pulses (KP's) in the hydranths. The coupling between the two systems is variable and determined by each hydranth.

The means by which the NN system induces activity in the ZNN is presumably junctional in nature. Consequently one might expect that the properties of each junction will largely dictate the manner in which the ZNN in an individual polyp will respond to NN pulses. In addition, the state of these junctions in different polyps might dictate how the whole colony responds to NN pulses. If all the NN-ZNN junctions are in the same state, all the polyps will withdraw simultaneously, if not they would respond with substantial individual variation. Both these responses occur. It is, however, important to remember that the way a colony responds to what is evidently the same stimulus may differ from time to time. This suggests that any variation there might be in NN-ZNN junctions in different polyps is not fixed but can vary temporally. Whether the variation is random or controlled by some component of the colony remains to be seen.

Variation in polyp responses to NN activity could also be explained by interneural facilitation within the ZNN system, and neuromuscular facilitation at the effectors. Although the recordings indicate that neuromuscular facilitation occurs (Fig. 5a, b, c) interneural facilitation was not observed. When ZNN pulses were recorded through two electrodes attached to different levels of the same polyp, the pulses appeared on both traces on a 1 : 1 basis and showed no signs of incremental spread.

Our evidence indicates that NN-ZNN junctions are at the top of the column of the autozooid. It can be seen in Fig. 4b that whereas the NN pulses are conducted up the column of the polyps, the ZNN pulses are always conducted downwards. This points to the interaction being at the top of the column, an argument supported by the behavior of decapitated polyps which are usually unable to withdraw or which are at least very late in withdrawing during general withdrawal over the whole colony. When stimulated directly these decapitated polyps can withdraw, indicating competency of musculature and ZNN systems.

Since it is suggested that the ZNN and NN pulses are the consequence of activity in separate nerve nets, it is implied that there are two physiologically separate nerve nets in the autozooids. As noted earlier, a well developed nerve net has been observed on the mesenteries of the autozooids (P. A. V. Anderson, in preparation) and has been implicated as being part of the neuromuscular system involved in polyp withdrawal and therefore the pathway for the ZNN system. The nerve net responsible for propagation of the activity that produces NN pulses has not as yet been identified. We are suggesting, however, that such a nerve net is present and separate from the Zooid Nerve Net.

In an ultrastructural study of *Renilla mülleri*, Spurlock and Cormier (1975) reported localization in the gastrodermal cells, of characteristic structures, luminelles, that contain what they believe to be lumisomes, the membrane-bound particles containing the essential components of luminescence (Anderson and Cormier, 1973). They suggest (Spurlock and Cormier, 1975, page 27) a control mechanism for luminescence involving either the transfer of calcium from nearby muscle fibers "as a result of excitation-contraction coupling" or else excitation of the luminescence by electrical activity conducted in muscle-cells. Assuming that the lumisome is the physiological source of luminescence and that it is identical with the ultrastructurally identified structure, it would still be doubtful that physiological luminescence control would involve a muscle cell intermediate.

In order for the first mechanism to operate, calcium must be released from the muscle cells in sufficient quantity to affect the luminescent cells. Assuming that some of the calcium involved in the excitation-contraction coupling does somehow escape to the extracellular fluid, that calcium must be made available to the luminelle. Normally, ionic calcium is avidly sequestered by cell membranes (Singer and Tasaki, 1968), mitochondria (Carafoli, Tiozzo, Rossi and Lugli, 1972) and endoplasmic reticulum (Ebashi and Endo, 1972) with the result that cytoplasmic calcium levels are typically lower than those of the extracellular fluid (Hasselbach and Makinose, 1972). If the calcium released by the muscles is to reach the luminelles, this sequestering must be reduced and the permeability of the luminescent cells to calcium must be high. If this is true in *Renilla* it is difficult to see how the luminescent cells could be protected from the high calcium levels found in the extracellular fluids (Hasselbach and Makinose, 1972) and still maintain both the general cellular metabolic activities that are inhibited by calcium (Lowe, 1970) and control over luminescence. The problem of extracellular calcium levels is the more apparent since the luminescent cells directly appose the coelenteron of the autozooid and will, at all times, be in direct contact with the calcium in the sea water.

With respect to the second proposal one would expect that electrical activity conducted through muscle cells would lead to contraction of those same cells. As we have shown, luminescence and contraction of autozooids are independent activities. Admittedly, polyp withdrawal would be expected to involve the mesenteric retractor muscles rather than the circular muscles that appose the luminescent cells (Spurlock and Cormier, 1975) so the observed independence could still occur yet allow the circular muscles to excite luminescence as suggested. However, *Renilla köllikeri* polyps are constantly active, displaying single tentacle contractions and extensions and column movements, all of which undoubtedly involve at least some circular muscle activity. If activity in these muscles is closely tied to luminescence, as is suggested in both the Spurlock and Cormier proposals, one would expect the individual autozooids to emit spontaneous luminescence. There have been no reports of such behavior in *Renilla köllikeri* and we have observed none. Perhaps variation in this behavior among the several species of *Renilla* is to be expected since spontaneous luminescence is said to be typical of *Renilla reniformis* (J. Wampler, personal communication).

The third conduction system detectable in *Renilla,* the Bp system, lacks behavioral and morphological correlates. Conduction in the Bp system is at a rate similar to that for NN pulses and pulses from it appear in all parts of the colony. The Bp system, like the NN system, is blocked by isotonic $MgCl_2$. Classification of the Bp system as a separate conduction system rather than a consequence of NN activity is based on the following. First, the NN conducts in an all-or-nothing manner. Pulses produced by each stimulus are propagated all over the colony with no decrement in size, although their size may vary with additional stimuli, probably as a result of facilitation of the recorded muscle action potential. However, varying the stimulating voltage should not change the effect of NN pulses throughout the colony. Consequently the fact that the stimulus threshold for Bp pulses is usually higher than that for the NN would indicate that they are different systems. Secondly, Bp pulses can be recorded after mechanical stimuli without preceeding NN pulses, as occurs following electrical stimuli. Finally, there are occasions when Bp pulses can be stimulated without the appearance of NN pulses (Figs. 3d, 4d).

The similar conduction rates in the NN and Bp systems are probably coincidental. Values in the order of 5–10 cm/sec have been recorded from numerous pathways in different coelenterates. The SS1 and SS2 conduct at rates similar to these in the sea anemone *Calliactis parasitica* and the NN pulses are conducted up the column wall at 10 cm/sec in the same animal (McFarlane, 1969a).

The results of our study suggest that in *Renilla,* there are two levels of conducting systems. One is confined to the individual autozooids and is responsible for polyp withdrawal, and perhaps other activities, in a manner that is reminiscent of the nerve net in sea anemones. The second level is that of colonial systems which seems to link effectors throughout the colony. The manner by which the one colonial conduction system, the NN system, controls the effectors is interesting. It has been suggested that rachidial contraction and luminescence are involved in escape behavior (Bertsch, 1968; Kastendiek, 1975) and would therefore be of great significance to the whole colony. Direct NN control of the effectors responsible for these activities is, therefore, not unexpected. On the other hand one might expect polyp withdrawal to be important to the autozooids individually, as well as to the colony as a whole. The indirect way by which polyp withdrawal is produced reflects this. The system allows a large degree of variability such as one might expect in an arrangement in which an outside influence, the NN pulses, tries to modify the behavior of a polyp which has the necessary machinery for independent activity.

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DIEL ACTIVITY RHYTHMS AND THERMOREGULATORY BEHAVIOR OF BLUEGILL IN RESPONSE TO UNNATURAL PHOTOPERIODS

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In reviews of Webb and Brown (1959), Cloudsley-Thompson (1961), Harker (1964), and Bünning (1967), it is apparent that among vertebrates, rhythms of birds and small mammals have received the greatest research attention.

Scattered reports, however, confirm the existence of daily periodicities in feedings and movements of several fish species in nature and under laboratory conditions [see Schwassmann (1971) and Beitinger (1974a) for references]. The majority of the observed daily periodicities of fishes appear to be cued by cyclic variations in light intensity and related to feeding behavior. However, extensive work by Barlow (1958) indicates that temperature is the critical stimulus in cyclic movements of the desert pupfish, *Cyprinodon macularis.*

Experiments (Beitinger, Magnuson, Neill and Shaffer, 1975) with green sunfish, *Lepomis cyanellus,* revealed the presence of a diel pattern in tunnel-pass frequency (*i.e.,* locomotor activity) of fish in our temperature-preference apparatus. Although simulated natural diel changes in day length directly affected locomotory activity, they did not significantly influence diel or hourly thermoregulatory performance of green sunfish.

In the following experiments two potentially disruptive, unnatural photoperiods were imposed to (1) determine the relationship between photoperiod and diel activity patterns, and (2) test the stability of thermoregulatory behavior of bluegill, *Lepomis macrochirus.* In particular, I looked for evidence suggesting the presence or absence of an endogenous component mediating activity patterns of thermoregulating bluegill.

Materials and Methods

Apparatus

The test apparatus is described in detail by Beitinger (1974a) and Beitinger *et al.* (1975). The design (Neill, Magnuson and Chipman, 1972) substitutes a temporal temperature gradient for the spatial gradient typical of most preferred-temperature studies and allows an individual fish to control its thermal exposure. Each 50-liter test tank is divided into halves with a molded fiberglass partition. A tunnel in the partition allows the fish to choose between halves differing by a fixed 2° C temperature interval. Movements through the tunnel are monitored by a pair of photoelectric cells. Passage of a fish into the warmer tank half causes the temperature of the *entire* tank to increase at a constant rate of 3° C/hr, while maintaining the constant 2° C differential between tank halves. When a fish swims into the cooler half of the tank, the temperature decreases at the same rate (3° C/hr) in both tank halves until the fish again moves to the warmer tank half. By moving back and forth in this manner, a fish is able to control the minimum and maximum temperatures to which it is exposed. For this study a potential temperature range of 4° C to 55° C was available.

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Illumination was provided by an incandescent bulb positioned above the center of each test tank. Bulbs were wired in parallel to a 24-hr timer joined to a capacitor-dimmer circuit. The latter provided a "soft on/soft off" light regime to simulate variations in light intensity which occur during dawn and dusk. Light intensity varied from about 1 footcandle during full illumination to 0 footcandles at full darkness.

Temperatures of each tank half were continuously monitored by a thermistor-wheatstone bridge circuit connected to a multi-channel analog recorder. Upper avoidance temperatures recorded as local maxima were computed from temperature records of the warmer half of each tank, while lower avoidance temperatures recorded as local minima were computed from temperature records of the cooler tank half. A local maximum was designated as an upper avoidance temperature if immediately preceded and followed by an interval when the warmer tank half was at least 0.5° C lower than the local maximum. Similarly, a local minimum was tallied as a lower avoidance temperature if preceded and followed by an interval when the temperature of the cooler tank half was at least 0.5° C higher than the local minimum. A mathematical description of avoidance (turn-around) temperatures was presented by Neill, Magnuson and Chipman (1972). The preferred temperature of each fish was operationally defined as the temperature midway between the two avoidance temperatures. Throughout the experiment, the number of passes through the tunnel by each fish, tallied by an event recorder, were utilized as a measure of fish locomotor activity.

Procedures

One fish was introduced per tank and allowed to experience the static system for 2.5 days with tank halves set 1.0° C above and 1.0° C below the fish's thermal acclimation state. The test period then began and tank temperature control was relinquished to each fish. Data collected during the first 24 hours were not analyzed. Two separate experiments were conducted.

In the first, bluegill, 87 ± 7.8 mm total length, captured during spring from Lake Wingra (Dane County, Wisconsin) were acclimated to 25° C and a LD 12:12 (*i.e.*, light/dark) photoperiod for 4 weeks. Nine fish were placed in the preference aquaria. Temperature and activity records collected during the second, third and fourth test days constituted control data. Following completion of the fourth day, photoperiod was switched from LD 12:12 to LD 6:6:6:6 and data were collected for an additional 72 hours.

Procedures in the second experiment were similar to the first, except bluegill (78 ± 6.2 mm total length), captured during summer from Lake Wingra, were initially acclimated to a LD 15:9 photoperiod and 25° C for 4 weeks. Following the 4-day control period (LD 15:9), a constant light regime (LL) was imposed on test fish and data were collected for an additional 96 hours. In both experiments, fish were fed pellets once each 24 hours on a random schedule during

TABLE I

Thermoregulatory performance of individual bluegill under control (LD 12:12) and experimental (LD 6:6:6:6) photoperiods. All values are temperatures in °C (sd equals standard deviation; grand mean equals the mean of the 7 individual means).

Fish	LD 12:12					LD 6:6:6:6				
	Avoidance temperatures				Preferred temperature	Avoidance temperatures				Preferred temperature
	Lower		Upper			Lower		Upper		
	Mean	sd	Mean	sd		Mean	sd	Mean	sd	
1	29.8	0.9	33.6	1.0	31.7	29.0	0.9	33.2	0.7	31.1
2	29.6	0.4	33.0	0.4	31.3	29.8	0.5	33.3	0.6	31.6
3	28.6	0.6	32.3	0.6	30.4	28.4	0.6	32.3	0.5	30.4
4	29.0	0.7	33.4	1.0	31.2	28.3	0.7	33.0	0.8	30.6
5	29.8	0.5	33.7	0.8	31.8	30.0	0.4	34.0	0.8	32.0
6	28.3	0.6	32.9	0.6	30.6	28.0	0.6	32.6	0.9	30.3
7	28.6	1.0	32.6	0.9	30.6	28.2	0.7	32.6	0.8	30.4
Grand mean	29.1		33.1		31.1	28.8		33.0		30.9

a light period. Pellets were introduced into the tank half occupied by each fish at the moment of feeding.

Locomotor activity and thermoregulatory performance data before and after photoperiod switch were compared. For each fish, the total number of tunnel passes each 0.5 hr (Experiment 1) and 1.0 hr (Experiment 2) was determined. The median for each 0.5 hr or 1.0 hr was plotted to present activity trends throughout both experiments. Also, hourly (or half-hourly) median activity data were pooled into 24-hour cycles for each photoperiod regime.

RESULTS

Temperature regulation

Two of the initial bluegill tested in the first experiment died owing to electronic failure. During the control LD 12:12 photoperiod, the remaining 7 bluegill had grand mean lower and upper avoidance temperatures of 29.1° C and 33.1° C, which yielded a preferred temperature of 31.1° C (Table I). During the two photoperiods, mean performance values of *individual* bluegill differed by 0.2 to 0.8° C (lower avoidance), 0.0 to 0.6° C (upper avoidance) and 0.0 to 0.6° C (preferred temperature). However, statistical comparisons of grouped differences yielded no significant trends in direction or magnitude for any of these thermoregulatory performance parameters (Wilcoxon matched pairs, signed ranks, all $P > 0.05$).

Thermoregulatory precision, reflected by the standard deviation of individual mean avoidance temperatures, also was not significantly influenced by the imposition of LD 6:6:6:6 photoperiod.

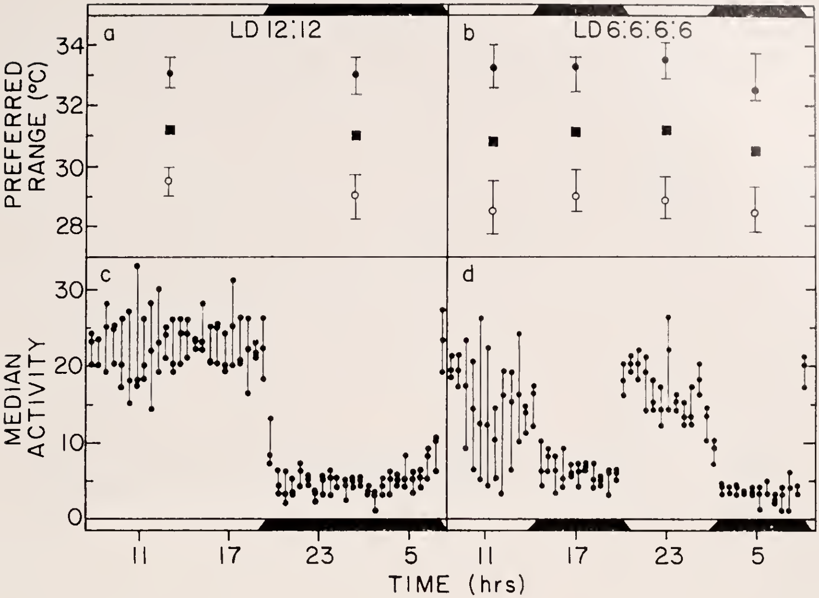

FIGURE 1. Thermoregulatory performance and locomotor activity of bluegill exposed to photoperiods of LD 12:12 and LD 6:6:6:6. Data collected during each photoperiod were pooled into their respective 24 hour cycles. Panels a and b show first and third quartiles and median lower (open circle) and upper (closed circle) avoidance temperatures calculated from data collection during each light and dark interval of both photoperiods. Preferred temperatures are represented by closed squares. Panels c and d present pooled median 0.5-hourly activity levels of the same fish.

Figure 1 (a and b) presents the preferred temperature range for each of the six light and dark intervals during this experiment. All individual avoidance temperatures generated during the six test days were pooled by the time of their occurrence into appropriate light or dark intervals. Then, median and interquartile lower and upper avoidance temperatures were determined and plotted for each light and dark interval. Preferred temperatures (represented by enclosed squares) ranged from 30.5° C to 31.2° C. Utilizing the two limits of the interquartile range (25th and 75th percentiles) and median (50th percentile) as representative data from each interval, a Kruskal-Wallis, one-way analysis of variance was performed. Results confirmed that neither the lower ($P = 0.43$) nor the upper ($P = 0.53$) avoidance temperatures varied significantly among the six intervals. The observed increase in variability of LD 6:6:6:6 data is partly attributable to differences in experimental time, *i.e.*, each interval in LD 6:6:6:6 encompassed only 15 total hours (with 1 hr twilight excluded, 5 hrs × 3 days = 15 hours for each of the light and dark intervals), whereas each of the two LD 12:12 intervals were of 33 hours duration (again, with exclusion of twilight, 11 hr × 3 days = 33 total hours). This resulted in large differences (N ranged from 16 to 112) in the number of avoidance temperatures generated by fish during the various light and dark intervals.

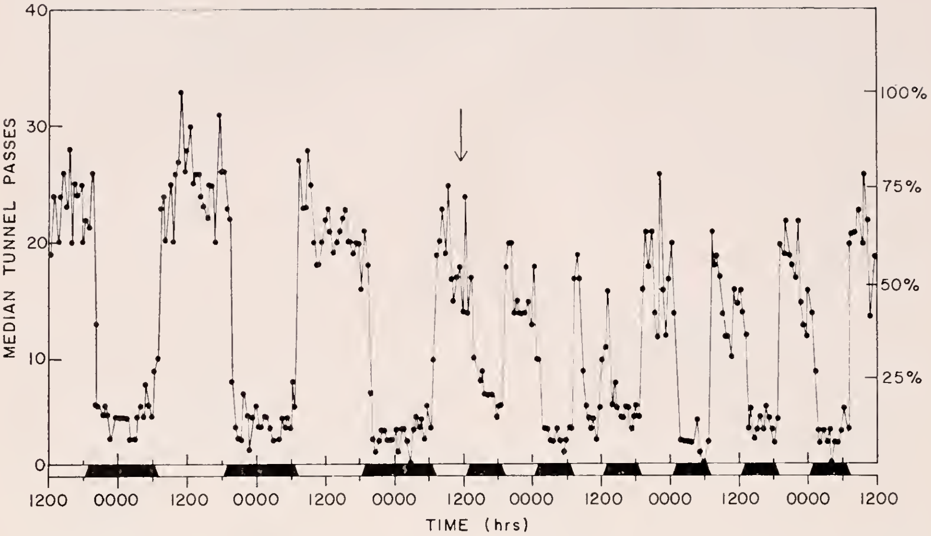

FIGURE 2. Median activity (0.5-hour, tunnel pass frequency) of 7 bluegill during the six day experiment. The arrow indicates when photoperiod was switched from LD 12:12 to LD 6:6:6:6.

Activity patterns

During the LD 12:12 (control) period, a distinct diurnal activity pattern was apparent (Figures 1c and 2). Day and night median activity levels (tunnel passes 0.5 hr) ranged between 15–33 and 1–10, respectively, and were highly significantly different ($P < 0.01$, Mann-Whitney U test). Fish demonstrated no obvious anticipation of dawn or dusk (Figure 1c). Activity levels varied sharply with illumination changes: activities increased during dawn and decreased following dusk.

To facilitate discussion of the LD 6:6:6:6 experimental period, the following time intervals were operationally defined: (1) abnormal dark—dark period from 1400–1900 hr; (2) normal dark—dark period from 0200–0700 hr; (3) abnormal light—light period from 2000–0100 hr; and (4) normal light—light period from 0800–1300 hr. Data collected during the hours when illumination intensity changed (*i.e.*, dawn and dusk) were omitted from analyses. In intervals 1 and 3 above, illumination levels were out-of-phase with the previous light/dark cycle; hence, responses during these intervals are of particular interest.

Activities of fish during the first abnormal dark period did not follow the typical "dusk response" in which activity decreases sharply with decreases in illumination (Figure 2). Median 0.5-hourly activity of fish during the first two out-of-phase dark intervals was increased significantly when compared to the activities of their successive in-phase dark intervals or any of the three LD 12:12 nights (Mann-Whitney U, $P < 0.01$). By the third 24-hour period of LD 6:6:6:6, however, in- and out-of-phase dark activities were no longer different (Mann-Whitney U, $P > 0.05$).

TABLE II

Thermoregulatory performance of individual bluegill under control (LD 15:9) and constant light (LL) photoperiods. All values are temperatures in °C (sd equals standard deviation; grand mean is the mean of the 8 individual fish means).

Fish	LD 15:9					LL				
	Avoidance temperatures				Preferred temperature	Avoidance temperatures				Preferred temperature
	Lower		Upper			Lower		Upper		
	Mean	sd	Mean	sd		Mean	sd	Mean	sd	
1	28.8	0.6	32.8	0.5	30.8	29.0	0.6	32.9	0.6	31.0
2	28.8	1.4	32.3	1.4	30.6	28.8	1.7	31.3	2.0	30.1
3	31.6	0.5	34.8	0.7	33.2	33.1	1.0	35.8	0.9	34.4
4	26.5	1.6	30.5	1.6	28.5	26.6	2.0	31.0	2.0	28.8
5	31.4	0.9	35.3	1.3	33.3	31.0	0.7	34.4	0.7	32.7
6	30.6	1.0	33.1	0.8	31.9	30.8	1.0	33.4	0.8	32.1
7	31.0	0.5	33.6	0.6	32.3	30.4	0.3	33.3	0.5	31.9
8	29.4	0.6	31.7	0.7	30.6	29.6	1.2	31.7	1.2	30.7
Grand mean	29.8		33.0		31.4	29.9		33.0		31.4

Comparing successive normal and abnormal light intervals, only in the first 24-hr period were activities significantly different (Mann-Whitney U, $P = 0.002$). Although a sharp increase in activity appeared when illumination levels increased, the sharp decrease in activity as illumination decreased was not apparent (Figure 1d).

Although significant differences were observed in activity levels of bluegill during light and dark intervals, there was no correlation between activity levels and avoidance temperatures (Spearman rank correlation, $r_s = 0.16$). Thermoregulatory performance of bluegill remained relatively constant.

Temperature regulation

During LD 15:9 photoperiod of the second experiment, the 8 surviving bluegill had grand mean lower and upper avoidance temperatures of 29.8° C and 33.1° C, and preferred temperature of 31.4° C. Switch to constant light conditions (LL) did not influence thermoregulatory behavior, as all three mean performance parameters were within 0.2° C of their respective control values (Table II). There were no significant trends in direction or magnitude of either avoidance temperatures of preferred temperature during LL when compared to those during LD 15:9 (Wilcoxon, all $P > 0.05$).

Although performance data for fish were more variable in Experiment 2, individual preferred temperatures during the four separate photoperiods of the two experiments were not significantly different (Kruskal-Wallis, one-way, analysis of variance, $0.70 > P > 0.50$).

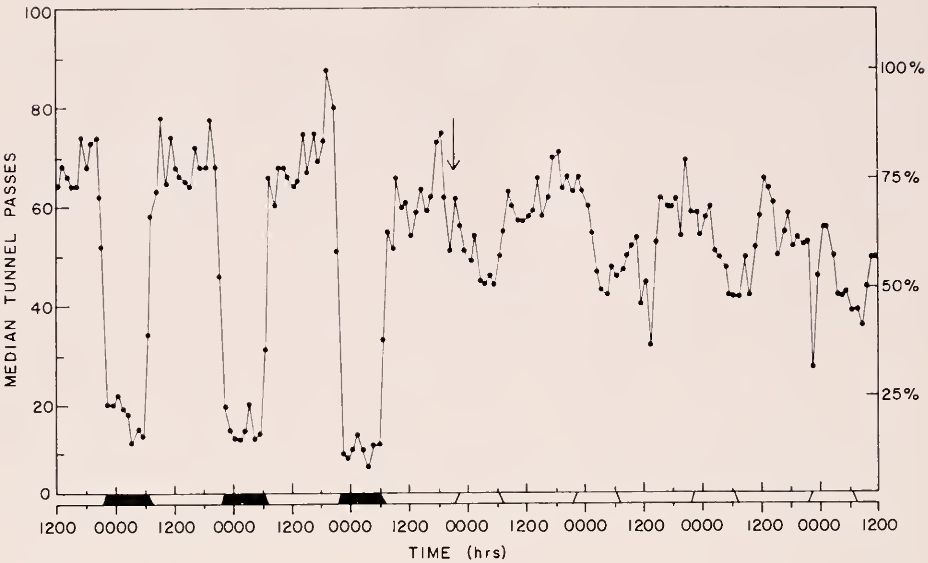

FIGURE 3. Median hourly activity of 8 bluegill exposed to a LD 15:9 photoperiod for three days and constant light for 4 days. The spaces between pairs of diagonal lines in the abcissa indicate when darkness would have occurred under the LD 15:9 photoperiod regime.

Activity patterns

The influence of simulated diel changes in light intensity on bluegill activity is well illustrated in Figure 4, formulated from pooled LD 15:9 activity data shown in Figure 3. Fish were highly active during the lighted portions, moderately active during the crespuscular times and least active during dark. Following switch to LL, the striking diurnal activity pattern observed under LD 15:9 was no longer apparent (Figure 3). During LL, day-time activity levels were lower and "night-time" activities were higher than those during corresponding times in LD 15:9.

A one sample run test (Siegel, 1956) indicated that the order of the 96 hourly activity medians during LL was not random, *i.e.*, a pattern existed ($Z = 6.78$, $P < 0.001$). Then periodogram analysis (Enright, 1965a, b) was applied to LL activity data. The resultant periodogram (Figure 5) has a broad spectral peak with a maximum amplitude between 24.5 to 25.0 hours, as well as an axis of symmetry near this time range. Although this analysis does not prove the existence of a circadian rhythmicity especially when applied to records of only four days, results suggest that a persistent rhythm was present in this data.

DISCUSSION

Preferred temperatures for bluegill in these experiments are consistent with those reported by Neill and Magnuson (1974) and Beitinger (1974b) using a similar experimental approach. Also, these values are within 1° C of the final temperature preferendum for this species determined in a vertical gradient of tem-

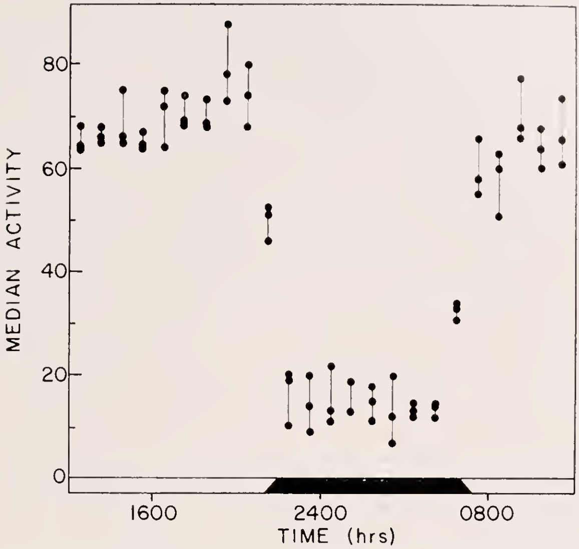

Figure 4. Hourly activity data of bluegill under 3 days of LD 15 : 9 pooled to show 24-hour rhythmicity.

perature by Fry and Pearson (1952). These laboratory-derived preferred temperatures approximate the deep muscle temperature and estimated temperature acclimation state of similar sized bluegill collected during daylight, in summer, from a thermal discharge area of Lake Monona (Neill and Magnuson, 1974).

Thermoregulatory performance of bluegill did not differ greatly between day and night (Figure 1a) or among 6-hour light and dark intervals (Figure 1b). The

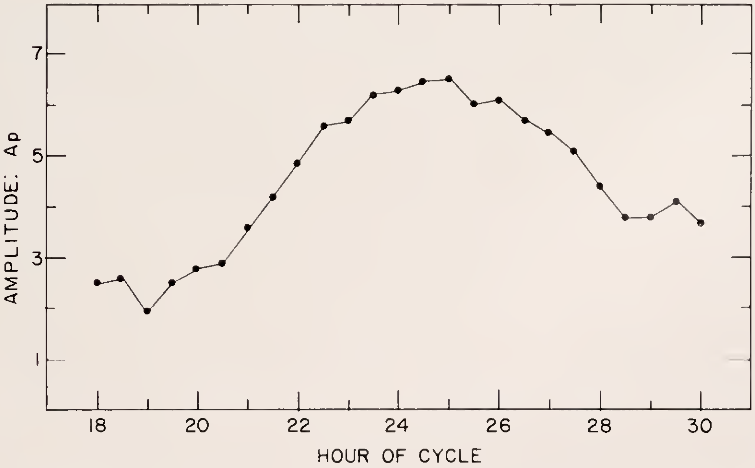

Figure 5. Periodogram computed from median hourly activity data of bluegill under 4 days of constant light.

night-time preferred temperature was 0.25° C lower than that during daytime; however, this difference does not approach the 2 to 3° C decrease in body temperature for night-captured bluegill reported by Neill and Magnuson (1974). This inconsistency in night preferred temperatures between laboratory and field data could be due to the intervention of natural non-thermal factors which are either controlled or absent in my laboratory system.

The influence of illumination on temperature selection of fishes has received little research attention. The mosquito fish, *Gambusia affinus*, was observed by Winkler (1973) to thermoregulate behaviorally only during daylight. Sullivan and Fisher (1954) found that temperature selection by brook trout, *Salvelinus fontinalis*, was more precise statistically in dim light (~ 0.15 footcandles) than in bright light (~ 15 footcandles), however, the mean and median preferred temperatures were the same (10.5° C) under both intensities.

The imposition of the two unnatural photoperiod regimes did not disrupt thermoregulatory ability or performance of test bluegill. Preferred temperatures of individual bluegill prior to and following photoperiod switches were similar. Of particular interest is the observation that preferred temperatures of fish under the four separate photoperiods were not significantly different. These findings attest to a stability in thermoregulatory behavior of bluegill.

Whether the realtionship between activity and thermoregulation in this experimental design is cause and effect (or effect and cause) is not known. Bluegill during the 2.5 day, temperature-static portion of Experiment 1 were also diurnally active. However, in comparing activity levels, these same bluegill were more than twice as active while thermoregulating. The low correlation ($r_s = 0.16$) between activity and preferred temperatures reveals that activity by bluegill does not exert a primary influence on the temperatures preferred. For a further discussion of the relationship between activity levels and thermoregulatory performance inherent in this experimental design, see Neill (1971) and Beitinger, Magnuson, Neill and Shaffer (1975).

In the control phase of both experiments, bluegill activity patterns were markedly diurnal (Figures 2 and 3). Each dawn, a sharp rise occurred in activity levels following a relatively inactive night. The increase in activity was maintained throughout the light period and then decreased rapidly with decreasing illumination during dark. This pattern for bluegill is similar to those of a variety of diurnally active fish species. In contrast to nocturnally active fishes which utilize tactile, chemical or electrical senses for food location, diurnally active species rely predominately on cone vision and typically are visual feeders, actively foraging for and capturing individual prey items.

Analyses of field captured bluegill from Lake Wingra (Baumann and Kitchell, 1974) support these generalizations. The mean stomach content of 75–95 mm bluegill (same size as those studied in these experiments) captured in September, increased almost linearly from a minimum at 0400 to a maximum at 1800. After 1800, mean stomach content decreased rather precipitously. Lake Wingra bluegill did not appear to feed at night. Thus, the periodicity of bluegill feeding in nature corresponds well with the activity pattern found in these experiments. Possibly, the high daytime activities by bluegill in the preference apparatus represent residual exploratory or food search behavior. Similar nocturnal depressions in feeding have been reported in other diurnally active species such as *Clupea harangus*

(Johnson, 1939), *Salmo salar* and *Salvelinus fontinalis* (Hoar, 1942), and *Salmo trutta* (Swift, 1964). Locomotor activity and feeding patterns of fishes are intimately associated and ecologically adaptive. In diurnally active species, decreases in prey abundance or capture efficiency occur simultaneously with inactivity during night, hence, hunger increases. Locomotor activity, which is an important element of feeding behavior, increases with increasing illumination at dawn.

Natural periodicities in daily illumination seem to be adequate in themselves to elicit the diel activity rhythm observed in bluegill; however, manifestations of an internal biological clock appeared in both experiments. In the first experiment, activity of bluegill exhibited resistance to LD 6:6:6:6 photoperiod entrainment, particularly during the first 24 hours (Figure 2). Activity levels during the first abnormal (out-of-phase) dark period were highly significantly increased over those of LD 6:6:6:6 normal (in-phase) and LD 12:12 dark periods. A similar disparity appeared in the relationship between activities during the first two 6-hour light periods. An endogenous component appeared to be reinforcing the responses to light and dark when they were in-phase with the previous light/dark regime and resisting those that were out-of-phase. However, by the third 24-hour period, no significant differences between in- and out-of-phase light or dark activity levels occurred, indicating bluegill had adjusted to LD 6:6:6:6.

The continuation of a bluegill residual activity periodicity in constant light (Experiment 2) provides more evidence to support the existence of an internal timing mechanism. Although the amplitude was dampened (Figure 3), a rhythm was apparent during the entire 96 hours of constant light. These results indicate bluegill possess or are able to develop an internal timing mechanism, synchronized to the external light/dark cycle. This timing mechanism permits the activity rhythm to continue when the external cues are absent for at least 4 days.

Retention of a pre-feeding response (increase in activity) by bluegill held under constant light (Davis and Bardach, 1965) also indicates the presence of an internal timing mechanism in this species. In contrast to a natural circadian rhythm, the pre-feeding activity rhythm is a learned response to a regular daily feeding. The presentation of food resets the timing of the internal clock each 24 hours. Since feeding was random in the LL experiment reported here, the observed activity rhythm was apparently free-running, possibly accounting for its decrease in amplitude with time. The repetitive pre-feeding response in LL (Davis and Bardach, 1965), the resistance of activity levels to immediate photoperiod entrainment and the continuation of a residual activity periodicity in constant light point to the existence of an internal biological clock in bluegill.

Although there have been few reports of persistent longterm activity periodicities of fish, several species have demonstrated evidence of an internal timing mechanism. Tomcod (*Microgadus tomcod*), scup (*Stenotomus vesicolor = S. chysops*), mummichog (*Fundulus heteroclitus*), largemouth bass (*Micropterus salmoides*) and bluegill held under 12 hour periods of bright and dim light acquired a pre-light, pre-feeding activity; also, mummichog established pre-feeding activity under constant illumination (Davis and Bardach, 1965). Recurrent periodicities in activity after removal of external cyclic stimuli have been reported in goldfish, *Carassius auratus* (Spencer, 1939), ammocoete and adult sea lamprey, *Petromyzon marinus* (Kleerekoper, Taylor and Wilson, 1961), common sole, *Solea vulgaris* (Kruuk, 1963), Atlantic salmon, *Salmo salar* (Ali, 1964), sockeye salmon, *Oncor-*

hynchus nerka (Byrne, 1968), a minnow, *Phoxinus phoxinus* (Müller, 1968), swell shark, *Cephaloscyllium ventriosum* (Nelson and Johnson, 1970), Gymnotid species (Schwassmann, 1971), and Atlantic herring, *Clupea harengus* (Stickney, 1972).

With the exceptions of the swell shark which maintained a circadian activity rhythm for about 18 days under both constant light and darkness and various species of Gymnotidae held under constant darkness, the activity rhythms of none of the above species persisted for more than 5 days after external cues were eliminated. Schwassmann (1971) and Richardson and McCleave (1974) suggest that activity rhythms may not always be good indicators of fish endogenous oscillators. Although more extensive records are required to assess the duration of bluegill activity periodicities in the absence of external light synchronizers, results herein indicate that activity patterns of bluegill in my thermoregulation apparatus are photoperiod entrainable and persist for at least 4 days under constant light conditions.

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SUMMARY

Locomotor activity patterns and temperature regulation of bluegill, acclimated to 25° C and 12-hour light/12-hour dark (LD 12 : 12) or LD 15 : 9 were examined prior to and following shifts to unnatural photoperiods. During the control phase of both experiments activity patterns of fish were typically diurnal. Activity levels varied sharply with changes in illumination intensity: activity increased during dawn and decreased following dusk. When photoperiod was switched to LD 6 : 6 : 6 : 6 (from LD 12 : 12) fish activity levels indicated a resistance to photoperiod entrainment, particularly during the first 24 hours. Following switch from LD 15 : 9 to constant light (LL), the striking diurnal pattern of activity observed under LD 15 : 9, was no longer apparent. However, a rhythm persisted throughout the entire 96 hours of LL. Results of both experiments suggest the presence of an endogenous component mediating bluegill diel activity patterns.

Switches to unnatural photoperiods were not disruptive to thermoregulatory behavior of bluegill in either experiment. Performance data collected before and after photoperiod switch in each experiment were similar. Mean preferred temperatures of grouped fish under the 4 different photoperiods (LD 12 : 12, LD 6 : 6 : 6 : 6, LD 15 : 9 and LL) ranged from 30.9 to 31.4° C and were not significantly different.

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REPRODUCTION AND LARVAL DEVELOPMENT OF *PSEUDOPOLYDORA PAUCIBRANCHIATA* (OKUDA) AND *PSEUDOPOLYDORA KEMPI* (SOUTHERN) (POLYCHAETA: SPIONIDAE)[1]

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Pseudopolydora paucibranchiata was described originally by Okuda (1937) from Onomichi, Hiroshima Prefecture, Japan. Since this find in the western Pacific it has been reported a number of times from the eastern Pacific. The earliest eastern Pacific report was as *Polydora* (?) n. sp. from Los Angeles-Long Beach Harbors, California (Los Angeles R.W.P.C.B., 1952). *Pseudopolydora paucibranchiata* was then identified by Reish (1954, 1955, 1959a, 1961a) and Barnard (1958) in surveys of pollution-related organisms in Los Angeles-Long Beach Harbor. Reish reported the species from Newport Bay (1959b) and from Alamitos Bay (1961b, 1963) in California. The species was overlooked, however, by Hartman (1969).

Pseudopolydora kempi was described by Southern (1921) from a brackish water lake in India. The original description was supplemented by Okuda (1937) who found the species in Japan. Imajima and Hartman (1964) designated the Japanese form as a new subspecies, *P. kempi japonica* and regarded the size, length of the caruncle, and number of branchial pairs as being characters of sufficient difference to warrant subspecific designation. The species was first reported from California by Blake (1966) and Reish and Barnard (1967) from Morro Bay. Light (1969) named a new subspecies, *P. kempi californica*, based on three specimens taken near Bolinas. A major distinction of *P. kempi californica* from *P. kempi japonica* was considered to be the absence of a nuchal tentacle in the former. Light also figured a rather unusual pygidium.

Pseudopolydora paucibranchiata and *P. kempi* are both common in sandy-mud sediments in bays and estuaries of central and northern California. The authors have collected *P. paucibranchiata* at Santa Barbara, Elkhorn Slough and Tomales Bay. *Pseudopolydora kempi* was found on mud flats in Morro Bay and was common at Lawson's Landing and Walker Creek in Tomales Bay where it occurred on sand and mud flats.

This paper describes the reproduction and larval development of *P. paucibranchiata* and *P. kempi*, with comments on developmental patterns of *P. antennata* Claparède and *P. pulchra* Carazzi. Some additional information on natural history is also provided. Systematics of *P. kempi* is reviewed in light of larval adult morphology, and a possible route of species introduction from Japan is suggested.

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METHODS AND MATERIALS

In the Tomales Bay studies, tubes containing adult worms were collected from sediments at Lawson's Flat and from the Walker Creek Delta. Tubes were opened and egg capsules were removed and examined under the dissecting microscope in the laboratory at the Pacific Marine Station. The stage of development of larvae within the capsules was determined and larvae were freed from the capsules for study.

Plankton samples were taken from the dock at Lawson's Landing, located near the mouth of Tomales Bay. During 1971–72 (September–August) samples were taken each week just before high tide. Polychaete larvae were removed from the sample and sorted to species.

Larvae were cultured in filtered sea water in Stender dishes. Water was changed every two days. Pure cultures of *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* were fed to the larvae. Constant temperatures were maintained in modified refrigerators and beverage coolers. Temperatures unless otherwise stated were at 17° C.

Worms collected by the second author at Morro Bay and Elkhorn Slough were transported to California State University, Fresno, where they were sorted and maintained in an air conditioned laboratory at 21° C. Larvae were removed from egg capsules, placed in culture dishes and observed. Larvae were fed a dried *Chlorella* powder.

It was decided to combine information and prepare a single paper from the two studies. Initial observations on larvae were compared and differences noted and discussed. For the most part, observed differences proved to be matters of interpretation and not actual differences due to studying different populations. Possible discrepancies were checked against actual specimens. In this manner we believe an accurate picture of larval morphology has been constructed.

Both authors used Zeiss optics with bright field and phase contrast. Nomarski phase interference was available to the first author. Line drawings were made from an initial observation and subsequently compared with other individuals at the same stage of development.

Pseudopolydora paucibranchiata (Okuda)

Reproduction

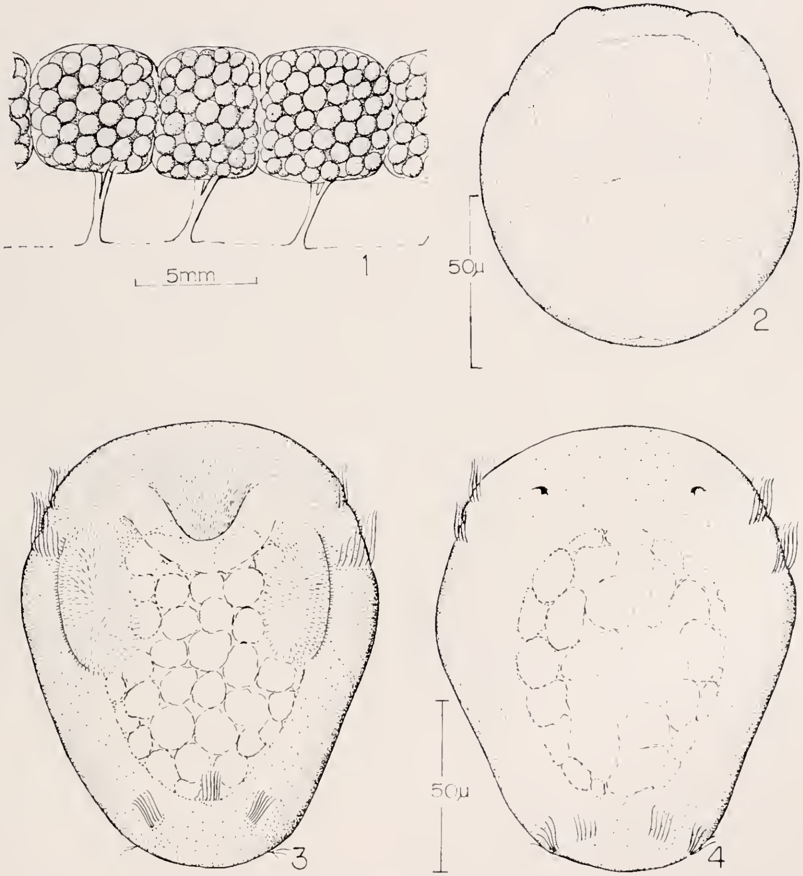
Although a year-round population study was not attempted, sexually mature adults were found in February and March (Elkhorn Slough) and from February to July (Tomales Bay). Egg capsules, however, have been taken in every month of the year in Tomales Bay. Pelagic larvae have been identified in Tomales Bay in each month except for January and February. The highest numbers of *P. paucibranchiata* planktonic larvae were encountered from July through October. Considering that larvae and egg capsules occur throughout most of the year, paucity of mature adults is undoubtedly an artifact of limited benthic collecting.

One mature male of about 45 setigers had testes and sperm present from setiger 24 through setiger 37. Another specimen with 60 setigers measured 12.0 mm, had branchiae on setigers 7–21, ripe gametes in setigers 21–45 and immature testes in setigers 46–60. A female of 42 setigers had branchiae on 7–16 and oocytes in setigers 15–30.

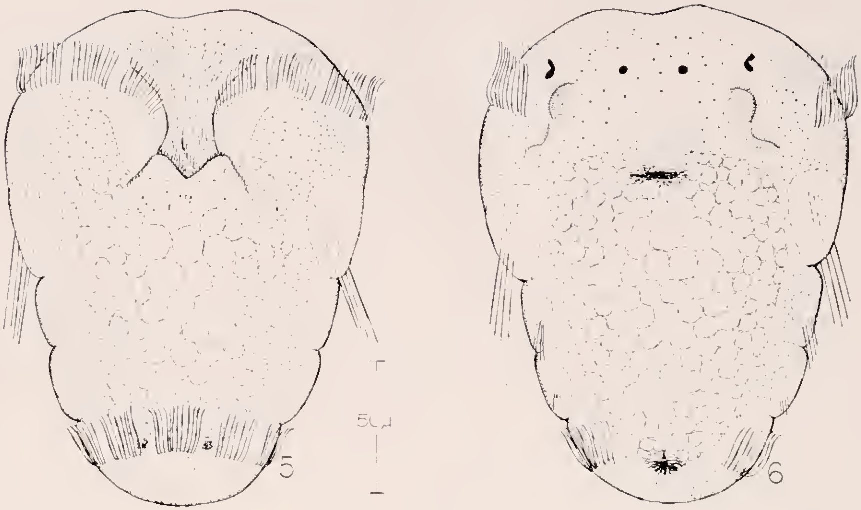
Eggs range from 96–105 μ in diameter but have a mean diameter of 96.2 μ. There are about 35–50 eggs per capsule and 7–10 capsules in a string. Each individual capsule is joined to the next and attached to the tube by two thin cytoplasmic extensions (Fig. 1). Mature sperm have the following measurements; total length 56.1 μ, acrosome 2.1 μ, nucleus 10.8 μ, middle piece 3.2 μ, and tail 40 μ. Sperm are of the long-headed type termed "aberrant" by Franzen (1956).

Development in the capsule

Development of a series of embryos from cleavage to free moving pre-setiger larvae takes about 24 hours. In the first five to six hours, development proceeds



FIGURES 1–4. *Pseudopolydora paucibranchiata:* 1) egg capsules; 2) early undifferentiated embryo removed from capsule; 3) pre-setiger larva in ventral view; 4) pre-setiger larva in dorsal view.

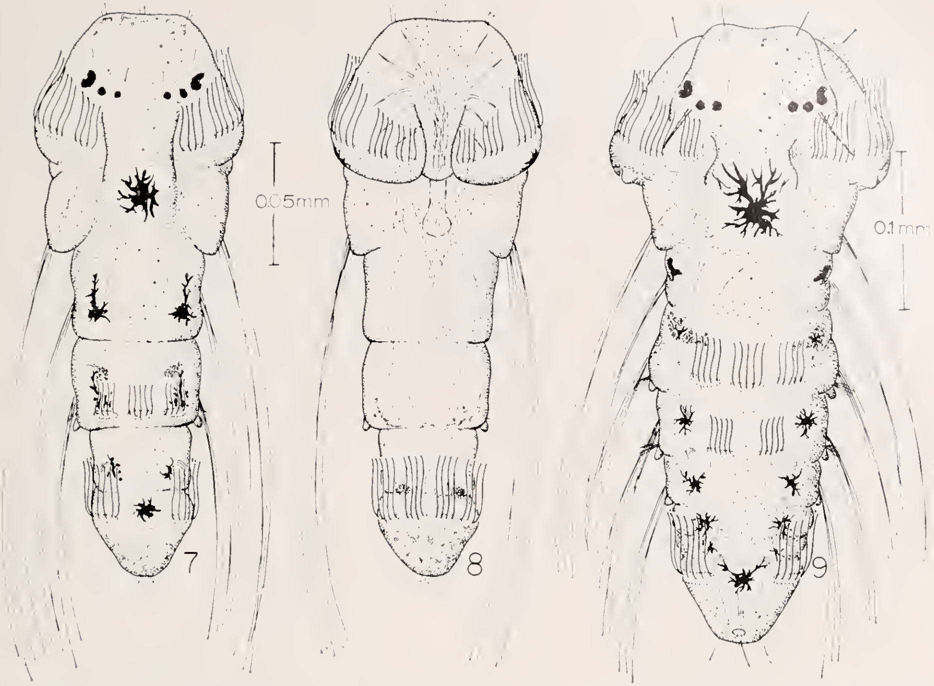


Figures 5–6. *Pseudopolydora paucibranchiata:* 5) early 3-setiger larva in ventral view; 6) early 3-setiger larva in dorsal view.

to a ciliated larva that rotates slowly in the sac. The youngest embryo removed from a capsule measured 102 μ at the widest point. Large yolky macromeres surrounded by micromeres are the only noticeable features. There is no evidence of ciliation (Fig. 2). At the end of 24 hours, the pre-setiger larvae are 130 μ in length (Figs. 3–4). The central region is filled with yolk. Two broad ventral ciliated patches are present. The prototroch has developed and is formed of two ciliated bands on either side of the anterior end. The anteriormost band is transitory but the posterior band continues to develop and is retained throughout the larval life. The mouth has developed and is heavily ciliated. The telotroch is a ring of seven isolated patches of cilia. There are two eyes located dorsally. There is no evidence of segmentation.

Early 3-setiger larvae (*Figs. 5–6*). Another 24 hours is required for development of the first three setigerous segments when the larvae measure 190 μ in length. The central yolk mass is prominent but the larvae have feeble swimming abilities when released from the egg capsules. The two ventral ciliated patches are gone. The prototroch has continued to develop and now ventrally merges with the cilia of the vestibule which has deepened. A broad neurotroch is forming posterior to the mouth. Cilia of the telotroch have lengthened. There is a dorsal gap in the telotroch. Four eyes are present; the inner pair is rounded; while the lateral pair is cup-shaped. Two ciliated areas on either side of the head represent presumptive nuchal ciliation.

There are two dorsal median chromatophores. One is located at about the level of setiger 1, while the second is on the anal segment. Two small patches of iridescent pigment occur ventrally on the anal segment. Dark pigment in the gullet is visible in dorsal view.



FIGURES 7–9. *Pseudopolydora paucibranchiata:* 7) pelagic 3-setiger larva in dorsal view; 8) pelagic 3-setiger larva in ventral view, posterior setae omitted; 9) pelagic 5-setiger larva in dorsal view.

Development in the plankton

Larvae emerge from the capsule with three fully developed setigers. Upon release from the capsule, larvae swim rapidly toward the surface and a light source, demonstrating a strong photopositive response. Soon their movement is more random with returns to the bottom of the culture dish to sweep it with their vestibule though the gut is as yet not complete. As they touch the bottom they flex their bodies and extend their setae, especially those of setiger 1.

PELAGIC 3-SETIGER LARVAE (*Figs. 7–8*). The hatching 3-setiger larvae range from 245–272 μ in length with a mean of 250 μ. The head is the widest part of the larva at 121 μ, with setiger 1 intermediate in width (95 μ), and the remainder of the trunk narrow (68 μ). The prostomium is definite in form only at the anterior end where it is broad with a slight medial indentation. The peristomium flares laterally and the lateral lips turn ventrally to the developing vestibule which although heavily ciliated has not opened to the yolk of the central intestine. The posterior end of the intestine is still separated from the anal tube of the pygidium. The anal tube is heavily ciliated. Later the vestibule and anal canal open to the yolky intestine and the feeding process moves food into and through the gut.

On the dorsal surface of the head there is a medial crescent of four small round eyes and an anterolateral pair of cup-shaped eyes. The prototroch is made up of five patches of cilia on each half of the head with the longest cilia in the lateral cells and the shortest bordering on the nuchal ridge and the vestibule. Nuchal cilia are short and difficult to see. The telotroch is formed of six groups of long cilia, with a dorsal gap. Stiff tactile cilia are present on the prostomium and posterior end. Nototrochs first develop on setiger 3. Lateral bundles of grasping cilia are also present. A neurotroch extends posteriorly from the ciliated mouth opening to the posterior border of setiger 1 and in some specimens to the middle of setiger 2. There is no ciliated pit.

The pigmented areas all appear dark to black with transmitted light, but in reflected light separate yellow and black areas can be differentiated. A pair of yellow iridescent pigment spots (reflected light) are found dorsolaterally on setiger 3 and ventromedially on the anal segment; paired spots are found on the extreme lateral surface of setiger 2 and dorsolateral on setiger 3. Spots occur on the sides of the peristomium. Granular rust-colored pigment is found on setiger 2.

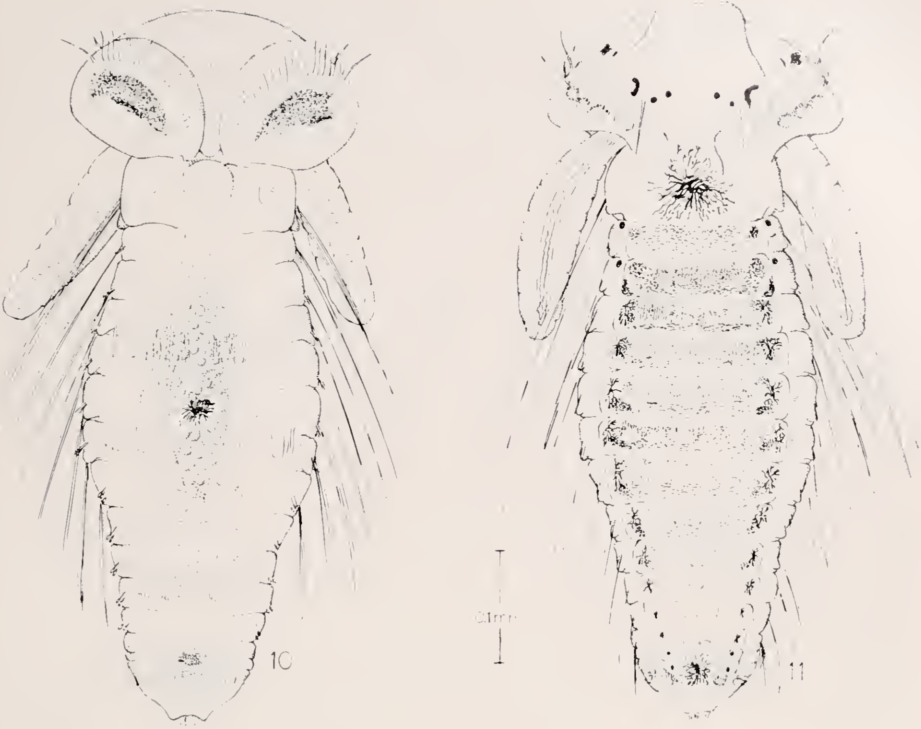
The larval setae are serrate. Those of setiger 1 extend beyond the pygidium and are about 200 μ in length. In setigers 2 and 3 they are successively shorter but still reach to the pygidium or beyond.

Pelagic 5 to 6-setiger larvae (*Fig. 9*). A five-setiger larva with two additional segments developing is about 375 μ in length. The head is wider than the body, measuring 163 μ, while at the level of setiger 3 the width is 100 μ. Setiger 1 is noticeably narrower than the head and from that setiger the body tapers gently to a rounded pygidium.

The prostomium has definite form anteriorly where it is wider than its less distinct tapered posterior end which reaches to setiger 1. The prostomium is gently notched anteriorly and is stippled with brown pigment. The peristomium flares laterally, due to elaboration of the lips of the vestibule. Palps are short; their development begins at about the 5-setiger stage. There are three pairs of eyes organized into two posteromedial pairs nearly in line and round in shape. An anterolateral pair is cup-shaped. Cilia of the prototroch are well-developed and very active. There is also a pair of stiff tactile cilia on the leading edge of the prostomium. The telotroch is made up of long active cilia forming a dorsally incomplete ring of six separate groups of cilia.

Nototrochs first appear on setiger 3 and continue on succeeding setigers. Grasping cilia occur on setigers 3–7. Gastrotrochs are found on setiger 5. The posterior end is stippled with brown pigment like the prostomium and bears three tactile cilia, two lateral and one medial. In addition to reddish-brown pigment on the anterior and posterior ends the other two types of larval pigment are also represented. Reflective yellow pigment is found in small rounded spots on the anterodorsal surface of the lateral oral flare of the peristomium and dorsolaterally on setiger 3 and as paired spots on the pygidium. Black pigment is represented dorsomedially by a large dendritic chromatophore on setiger 1 and a smaller one on the anterior margin of the anal segment. The black pigment also occurs in paired small black patches on the posterior margin of the peristomium and paired dorsolaterally on segments 2–6 and on successive segments as they develop. The gut has a yellow-green color due to ingested food, especially in the anterior part of the intestine.

Setal sacs of setiger 1 are large and the larval setae extend to near the posterior end. Setae of setiger 2 reach to the anal segment and each succeeding



FIGURES 10–11. *Pseudopolydora paucibranchiata:* 10) pelagic 13-setiger larva in ventral view: 11) pelagic 13-setiger larva in dorsal view.

segment has shorter setae. Notopodial lobes are distinguishable on setigers 3, 4 and 5. Stiff sensory cilia are associated with the lobes.

Larvae swim near the surface of the water and edge of the dish but regularly move to the bottom. There they maneuver in loop-the-loop fashion scraping their oral area along the bottom in an effective feeding activity. They tend to swim with posterior end bent down and the long larval setae angled upward.

Larvae with eight setigers remain near the bottom with greater regularity and they also often swim ventral side up.

PELAGIC 13-SETIGER LARVAE (*Figs. 10–11*). A 13-setiger larva measures 640 μ in length and is fusiform. The head width is greatly increased by expansion of the lateral lips of the vestibule. Palps arise from the dorsolateral region of the peristomium. Each palp has a ventral ciliated groove. The six eyes are similar in arrangement to those of the 6-setiger stage. A nuchal ridge is present with areas of rapidly beating cilia on either side. The prototroch is well-developed with several long tactile cilia emerging from various locations. Nototrochs begin on setiger 3 and continue on posterior setigers. Setiger 3 has four patches, setigers 4–8 have six patches and setigers thereafter have four again. Gastrotrochs occur on setigers 3, 5, 7, and 11. Those on setiger 3 are small and often lost at this stage. There are four patches on setiger 3 and six on 5, 7, and 11. The telotroch, which is composed of several patches of cilia, encircles the anal segment, but has a dorsal gap. The neurotroch extends halfway into setiger 1. Several small patches of cilia occur lateral to the neurotroch on the ventral side of setiger 1.

The pigment is fully developed and follows the pattern established in the 6-setiger stage. The dorsal surface of setigers 2–11 is covered with a reticulate green pigment. Black pigment is heavy on the ventral side of the peristomium. Light brown, non-reflective pigment occurs on the anal segment and on the margins of the peristomium and lips of the vestibule. The cilia of the telotroch occur in unpigmented areas. Two medial chromatophores occur ventrally; one is on about setiger 6 and the other on the anal segment. The former is black while the latter is iridescent yellow in reflected light.

Two prominent areas of reflective yellow pigment occur on the dorsolateral aspect of the peristomium. Similar reflective pigment occurs dorsally with the lateral chromatophores from setigers 3–10. As larvae of this stage swim, their posterior end is held lower than the prostomium, and their elongate palps trail posteroventrally and may be recurved with tips anteriorly directed.

Settling, growth, and metamorphosis

Larvae settle after the 13-setiger stage and before the 18-setiger stage. One specimen which settled prior to the 17-setiger stage added eight setigers in 15 days and another added nine setigers in 37 days. Growth rates are dependent upon temperature. Studies relative to growth rates are in progress and will be reported in a later paper.

Juveniles with 23 to 26 setigers measured 2.8 to 3.0 mm. Specimens with 46 to 49 setigers were approximately 5.0 mm long. The youngest individual noted to possess sexual products was a female of 42 setigers which had branchiae on setigers 7–16 and oocytes in setigers 15–30. The largest individual reared beyond settling was 12.0 mm in length and 60 setigerous segments. This specimen had branchiae on setigers 7–21 and mature gametes in setigers 21–45.

Metamorphosis is accompanied by loss of larval setae, pigment and most ciliary bands. The head becomes elongated and the palps are directed forward; they grow rapidly and acquire regularly spaced pigment spots along their length. The spots are conspicuous and appear yellow by reflected light. They are largest distally and decrease in size towards base. As the juveniles grow there is a regular increase in the number of spots on the palps as well as an increase in the number of branchiae.

TABLE I

Relationship of palpal pigment spots and number of branchial pairs to segments in Pseudopolydora paucibranchiata *adults.*

Number of segments	Number of pigment spots on palps	Setigers with branchiae
18	1	7–9
22	3	7–10
23	5	7–11
27	8	7–12
42	10	7–16
49	12	7–18

By counting the number of palpal pigment spots and the number of gills it is possible to estimate the size of the worm. Since the palps and the anterior end of the animal regularly protrude from the tubes which they occupy one can make counts without having to remove the worm from the tube and, thus, alter its behavior pattern. It is also possible to determine more closely the size of incomplete worms by utilizing the palp pigment and/or branchial counts (see Table I).

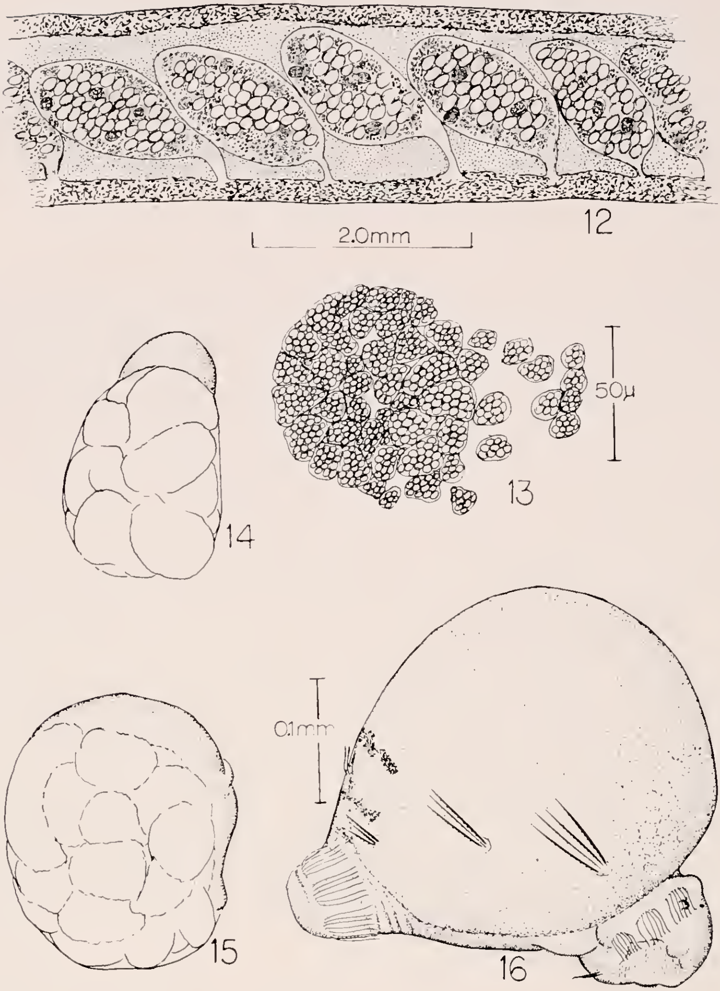
Natural history

In Elkhorn Slough, *Pseudopolydora paucibranchiata* was collected in association with *Phoronopsis viridis* Hilton and *Streblospio benedicti* Webster in a sandy habitat. They occurred in a zone lower intertidally than *Boccardia proboscidea* Hartman. The tubes of *Pseudopolydora paucibranchiata* are constructed of silt and/or fine sand with firm inner walls which have a spiraled pattern of construction. This pattern provides greater strength, and in addition, if the tube is broken, the broken end tends to curl and close off the tube. Because the tubes are constructed with fine sand or silt as they are available, the tubes have alternating sections of wall materials. Most tubes have multiple openings. Tube construction may be very rapid, for one specimen added 2.0 mm of tube in 5.0 minutes but a more normal construction pattern was the addition of 9.0 mm in 12 hours. The rate is in part dependent on the availability of foods and tube materials.

Tube construction proceeds in the following manner. The first three setigers of the body are extended from the tube and the active palps sweep silt along the ciliary groove to the basal region where it is gathered around the mouth. The worm then bends the anterior end down over the edge of the tube and touches it with the ventral side of the peristomium and the mouth region. The anterior end is then drawn back into the opening of the tube and the silt in the mouth area is added with a smooth downward sweep of the mouth from the new section towards the inner lining of the tube. This process is repeated around the periphery of the tube lip both clockwise and counterclockwise. The palps move massive amounts of silt and during this period of observation 2.0 mm of tube were constructed in 5.0 minutes as noted above.

Fecal strands are deposited about 5.0 mm away from the tube opening. The strands, measuring 2.0 mm or longer, regularly break up into elongate pellets having an undulating conformation easily distinguishable from the straight rod-like fecal pellets of an associated polychaete, *Streblospio benedicti*. Fecal strands are not deposited external to tube by extending the pygidium but are moved from tube entrance by the palps.

One adult in a silt tube was placed in a dish with half the substratum of silt and the other half of fine sand. At 0.0 hours the posterior half of the worm was in the tube and the anterior extended. Twenty minutes later it had left the tube and was crawling in the sand; at 30 minutes, 90 minutes, and 16 hours it was still in the sand. At 40 hours it had produced a sand tube, but with the newest section made of silt; the tube was 20.0 mm long. At the end of $4\frac{3}{4}$ days the tube was mainly silt and measured nearly 40.0 mm. The adults may construct the original and basal portion of the tube from sand but then in processing silt in feeding they add to the tube mainly from the silt debris.



FIGURES 12–16. *Pseudopolydora kempi:* 12) egg capsules in tube with contained embryos, nurse eggs and fragmented nurse egg granules; 13) nurse egg in the process of fragmenting into discrete granules; 14 and 15) early embryos prior to engulfing yolk granules; 16) encapsulated 3-setiger embryo after eating nurse egg granules and beginning morphological differentiation.

Pseudopolydora kempi (Southern)

Reproduction

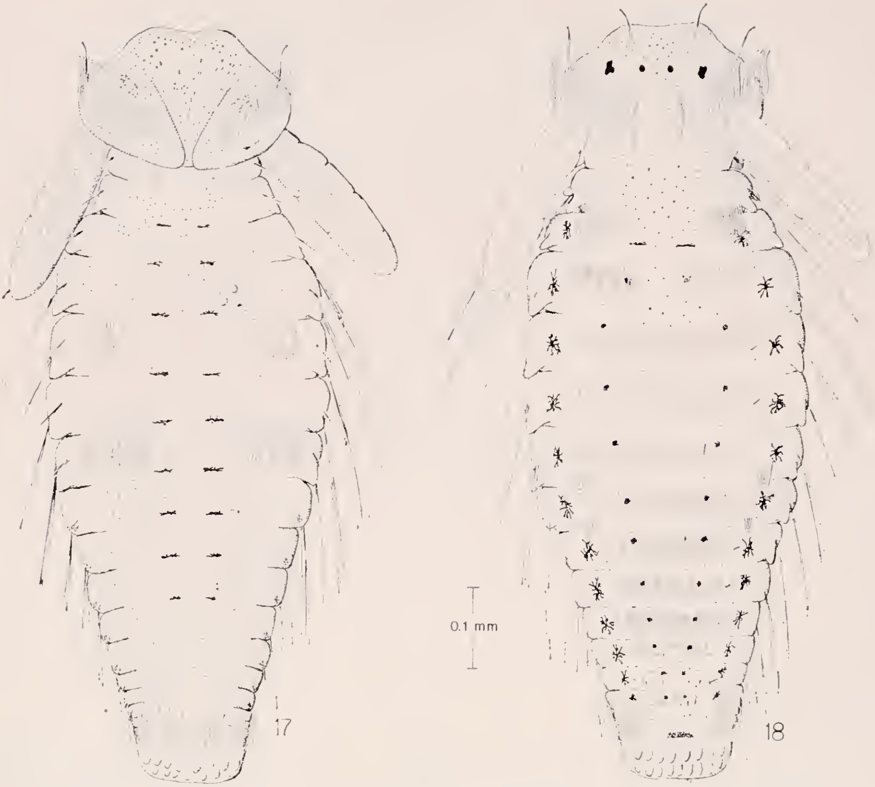
Although a year-round population study was not attempted, mature males were taken mid-February at Morro Bay and in October and November in Tomales Bay. Mature sperm have the following measurements: total length 57.5 μ, acrosome 0.8 μ, nucleus 7.5 μ, middle piece 4.6 μ, and tail 44.6 μ. Egg capsules were collected from tubes of adults in Tomales Bay from August to April and at Morro Bay in July. Ten to sixteen capsules were found joined together in loosely connected chains (Fig. 12). Each egg capsule is pear-shaped and connected to the inner lining of the tube by a thin extension of capsular material. The capsule shape is nearly identical to that of *P. antennata* as described by Rasmussen (1973). There are 15–20 capsules in a string. In each capsule the majority of eggs are unfertilized and serve as food (nurse eggs) for a few developing embryos. The nurse eggs range in diameter from 150–200 μ (mean = 165 μ). Early in development, however, the nurse eggs readily break up into yolk granules and at that time are difficult to count and measure. Developing embryos number up to 20 per capsule and measure 270–300 μ in diameter at an early stage.

Development in the capsule

Early in development the capsule is crowded with intact nurse eggs, yolk granules and a few embryos. The nurse eggs soon break up into separate yolk granules (Fig. 13). The earliest embryos removed from the capsule consist of large internal macromeres and a covering of unpigmented micromeres (Figs. 14–15). These simple embryos rapidly develop a ciliated vestibule and begin to ingest the yolk granules, storing them within their body. Ingestion is continued until all extrinsic yolk is taken. The embryos are essentially undifferentiated at this time, being merely a mouth and a mass of yolk. The distended embryos with contained yolk now occupy all of the capsular space formerly occupied by the embryos and nurse cells. Morphological differentiation commences with development of cilia and setae.

THREE-SETIGER LARVAE (*Fig. 16*). Larvae with three setigerous segments measure approximately 300 μ in length and 270 μ in width. They have a large ciliated vestibule, several patches of prototrochal cilia, and a telotroch. Three sets of serrate larval setae on each side mark the location of future segments. Ventral cilia are present at the level of setiger 1. Nototroch cilia are visible at about setiger 3. Several areas of black granular pigment are present dorsally near the posterior end of the larva. The pygidial area appears golden brown in transmitted light. Encumbered by the enormous yolk supply the larvae were incapable of swimming when released from the capsule at this stage.

EIGHT-SETIGER LARVAE. Larvae with eight setigerous segments have a well-developed prostomial-peristomial region which is gently rounded in shape. There is a pair of tactile cilia, one on each side of the midline, which project anteriorly from the leading edge of the larvae. The palps resemble elongate knobs. There are two pairs of eye spots in contrast to the 6–7-setiger stage which has only a single central pair. Dorsal pigment consists of two lateral rows of spots and two central rows on setigers 3, 4, 5, and 6. There is diffuse central pigment on setiger 7 and a well-developed dorsomedial pigment spot on the anterior margin of the anal segment. The larva is somewhat enlarged in its middle segments and the contained yolk mass has an hour glass shape. The yolk may bulge the body and distort segmental lines. Long larval setae project from the first four or five setigers. Those of the first setiger are longest and reach to about setiger 6 or 7. Setae on setigers 6 and 7 are very short. The posterior end of the body is gently tapered in shape and the anal segment is rounded.



Figures 17–18. *Pseudopolydora kempi:* 17) pelagic 15-setiger larva in ventral view; 18) pelagic 15-setiger larva in dorsal view.

Ten to 11-setiger larvae. At this stage the larval form is generally elongate with a tapered posterior end and a rounded anal segment. The anterior end is squared off medially and the four eyes are aligned nearly straight across the body just anterior to the palpal bases. The central pair are smaller and round; the lateral pair are larger and polymorphic. The palps are more elongate than in preceding stages and reach to the midline of setiger 2. The four rows of dorsal pigment are formed by spots on setigers 3, 4, 5, 6, 7, and 8. The spots are less distinct and pigment is diffusely distributed dorsomedially on setigers 9 and 10. The anal segment is pigmented as before. There is a pair of large pigment spots ventrally placed in the vestibular region. Larval setae are present. The modified fifth setiger is not specialized at this stage.

Twelve to 13-setiger larvae. Larvae are brooded in the capsule until they have at least 12 setigers. During this period the larvae are distended with yolk and until release they subsist entirely on yolk. The digestive tract is not complete posteriorly until the very end of the brooding period. The length of time spent in the capsule probably depends on the number of nurse cells originally available.

At about the 12-setiger stage and release from the capsule, larval setae are lost and short adult notosetae form on anterior setigers. Neuropodial lobes also form at this stage. These larvae when freed from the capsule may swim actively for hours or several days or may move directly to the bottom and spend most of the time slowly moving along the bottom and feeding on silt and other debris in the laboratory culture dishes. They also fed on less successful, decomposing larvae present in the dishes but this may be unnatural laboratory-influenced behavior.

A larva artificially released from a capsule at the 12-setiger stage developed the thirteenth setiger in 48 hours. It had four rows of dorsal pigment on setigers 3–9, two rows on setigers 10–12, and a single median pigment spot on setiger 13. Also noticeable at this stage are the two rows of ventral pigment on setigers 2–8.

Development in the plankton

PELAGIC 15-SETIGER LARVAE (*Figs. 17–18*). Large pelagic larvae were encountered in Tomales Bay plankton in March 1970 but they were never as abundant as *Pseudopolydora paucibranchiata* larvae. Pelagic larvae of *P. kempi* at this stage measure 850–900 μ in length. Their body is somewhat expanded centrally producing an overall modified fusiform shape. The prototroch extends ventrally to near the lips of the vestibule and dorsally covers about two thirds of the head. The low nuchal ridge is surrounded on each side by a few short nuchal cilia. A short neurotroch extends posteriorly into setiger 1. Four tactile cilia arise from the head, two located in front of the eyes and two located laterally near the prototroch. Palps reach into setiger 4. There are four eyes; a round inner pair and an irregular shaped lateral pair. Parapodial lobes are well-developed. Serrate larval setae may still be present on each segment including setiger 5. Gastrotrochs occur on setiger 5 (two patches) and setiger 7 (four patches). Nototrochs begin on setiger 3 and continue on succeeding setigers. Nototrochs of setigers 3 and 4 do not extend all of the way across the segment. Grasping cilia begin on setiger 5. The telotroch is formed of six discrete patches of cilia separated by a dorsal gap. The anal segment has golden brown pigment as does the prostomium. The anal segment also contains glandular structures. Branchial buds occur on setigers 7 and 8. The dorsal pigment consists of two lateral rows of branching chromatophores which begin on setiger 3 and continue on succeeding segments. There are also two central rows of pigment spots which are more granular in nature. These usually begin on setiger 3 but may not appear until setigers 4 or 5. There is a centrally located black pigment spot on the dorsal surface of the pygidium. Ventral pigment begins on setiger 2 and consists of paired bars on the posterior border of each segment. Some irregular areas of black pigment occur above and below the prototrochal cilia on the ventral side of the larva. The anterior margin of the prostomium and the posterior end have considerable golden brown pigment. The gut is packed with oily globules and digestive residue of phytoplankton. The anal segment bears gland-like papillae on its surface.

Growth, settling and metamorphosis

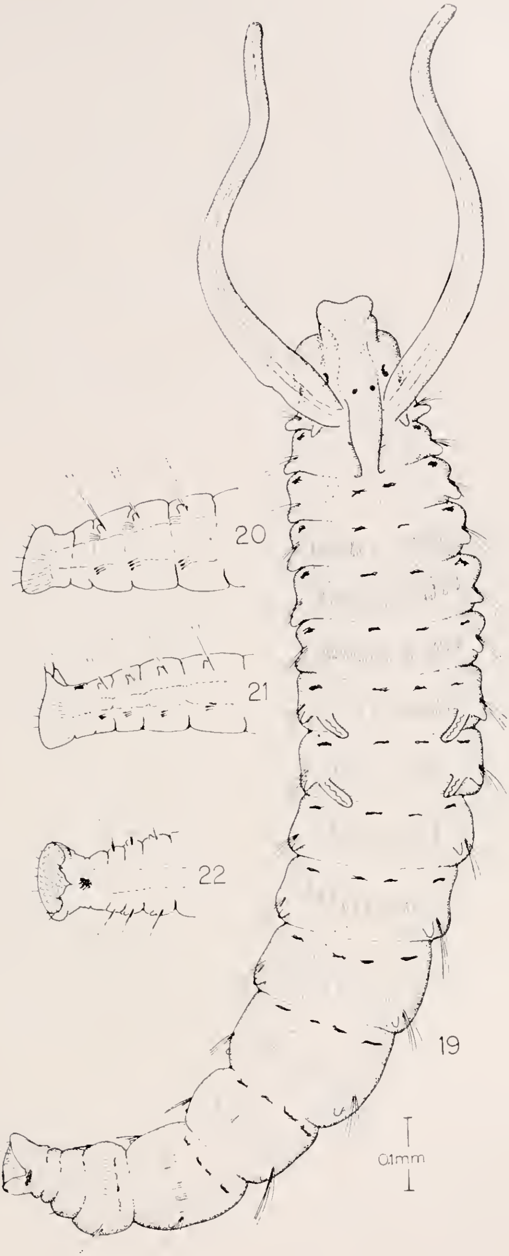
One larval specimen released from an egg capsule and observed regularly over several days in a laboratory culture dish was maintained at about 21° C. It was at 10 setigers on day 1, 12 setigers on day 2, and 14–15 setigers on day 3.

A 12–13 setiger larva freed artificially from a capsule collected at Morro Bay was placed in a culture dish of sea water with silt from the adult habitat. The larva immediately accumulated silt on its body and crawled through the silt, abandoning all swimming activities. In the next 72 hours its settling activities included tube construction. The dorsal body pigmentation was somewhat reduced but the basic pattern was retained. In 24 more hours the golden brown anterior pigment had become more noticeable. Palps elongated, became prehensile and reached about two-fifths the way back along the body.

Young adults placed in culture dishes containing sand, mud, and silt from the habitat produced extensive tubes with as many as six or seven openings. The tubes were of silt and mucous with an outer covering of coarser sand grains. The adults were secretive compared with *P. pacibranchiata* and rarely appeared at the tube openings. They extended the palps only to feed or dispose of fecal materials. Adults have white spots on the palps. The spots are numerous and irregularly arranged. One large adult collected at Morro Bay had 50 segments and measured about 13.0 mm.

Metamorphosis of the pelagic larva into a benthic juvenile involves loss or modification of larval organs and development or elaboration of other organs which have not been functional during the larval phase. Chief among the latter structures are the palps which do not process food during larval life but become the main organ of feeding during benthic life. In a settled 12-setiger specimen the palps, even in a slightly contracted state, reach to setiger 8. In conjunction with development of the palps there is a marked elongation of the prostomium and peristomium and loss of larval cilia. A 16-setiger juvenile is figured (Fig. 19). The prostomium projects anteriorly and is clearly bifurcate; posteriorly it reaches to the position of setiger 1 or the middle of setiger 2. The eyes have assumed a more typical adult arrangement, with a closely spaced oval posterior pair and a larger anterior pair, irregularly shaped and widely separated. Most of the larval pigmentation is retained in the juveniles. As juveniles grow, however, the lateral pigment bands on the anterior segments elaborate and spread down the sides of the segments. The strong anterior bands remain and become part of the adult pattern, but other pigment disappears. Lateral pigment may develop in setigers 1 and 2. Larval setae have been lost and adult setae are present in all but the incipient setigers of a 12-setiger stage. Setiger 5 is not highly specialized but the internal formation of spines can be discerned. Hooded hooks are present in setigers 8, 9, 10, and 11. The digestive tract is complete and the interface of esophagus and intestine is located at the anterior margin of setiger 6. Numerous sensory cirri are distributed on the palpal and pygidial surfaces. Changes in the pygidium are of interest. Immediately following metamorphosis the pygidium is a simple cup-shaped structure similar to that of *P. paucibranchiata* and many species of *Polydora* (Fig. 20). With continued growth, however, two dorsal elevations or projections appear on the previously simple pygidium (Figs. 21–22).

A metamorphosing form with 19 setigers and three incipient segments taken in August at Morro Bay had a caruncle which reached to the middle of setiger 3. Eyes were as before and no nuchal tentacle was observed. Palps reached to setiger 11. Five modified setae were present on each side of setiger 5. Branchiae began



Figures 19–22. *Pseudopolydora kempi:* 19) benthic juvenile with 16 setigers; 20) pygidial structure of newly settled juvenile in lateral view; 21 and 22) pygidial structure of older juvenile in lateral (21) and dorsal (22) views; 20–22) not to scale.

on setiger 7, were full size on 8 and 9, were small on 10 and 11. Numerous other juveniles were taken in mid-December in a benthic sample.

Natural history

In natural habitat members of this species form rust-colored tubes. Adults maintained in the laboratory in silt constructed extensive dendritically arranged tubes with as many as six or seven different openings. Branched tubes were not observed in the field, however.

In Morro Bay, *Pseudopolydora kempi* was collected in association with *Boccardia hamata* (Webster), *Polydora nuchalis* Woodwick, *Streblospio benedicti, Armandia brevis* Moore, and *Hemipodus californiensis* Hartman.

In Tomales Bay, *P. kempi* occurs on tidal flats in mixed sand-mud sediments The species is often associated with *Pseudopolydora paucibranchiata, Hemipodus borealis* Johnson, *Capitella capitata* Fabricius, *Pygospio elegans* Claparède, *Eteone dilatae* Hartman, and *Armandia brevis.*

Discussion

Information pertaining to larval development of spionid polychaetes is extensive and has been reviewed by Hannerz (1956), Simon (1967) and Blake (1969). Most larval studies of this family have concerned organisms from Europe or from the east coast of North America. Larval studies of species from the west coast of North America include those of Hartman (1940, 1941) on *Boccardia proboscidea,* Woodwick (1960) on *Polydora nuchalis* and Dean and Blake (1966) on *Boccardia hamata.* To date there is little published information on reproductive habits of any species of *Pseudopolydora* and no information on the larva development of *Pseudopolydora paucibranchiata* and *P. kempi.*

Pseudopolydora paucibranchiata and *P. kempi* exhibit very different modes of early development and brood protection. *Pseudopolydora paucibranchiata* has small eggs (96–105 μ), no nurse cells, larvae emerge from the capsule at the 3-setiger stage and spend a long period of time in the plankton. This pattern, with a short period of brood protection followed by a long planktotrophic period is typical for most related species of *Polydora* and *Boccardia* (Wilson, 1928; Dean and Blake, 1966; Blake, 1969). Seven species of *Polydora* and one of *Boccardia* have, however, been shown to have nurse cells and to spend little or no time in the plankton (Blake, 1969). *Pseudopolydora antennata* has been shown by Rasmussen (1973) to develop without nurse cells.

The nurse cell feeding pattern of *Pseudopolydora kempi* is very unusual but similar to that of another spionid, *Pygospio elegans* (Hannerz, 1956; Rasmussen, 1973). Within a single capsule the many nurse cells are fragile and immediately disintegrate into small yolk granules. The few embryos develop a vestibule and engulf the granules. When these are all eaten, the larvae are greatly distended with the enormous yolk supply which they utilize during growth and development in the capsule. The nurse cell feeding pattern of *Pseudopolydora kempi* differs markedly from that of *Polydora quadrilobata* Jacobi described by Blake (1969). In *P. quadrilobata* the nurse cells remain intact until they are engulfed whole by the larvae, which do not feed until about the 4-setiger stage. They con-

TABLE II

Patterns and distribution of pigmentation in four species of Pseudopolydora.
(– *indicates pigment absent;* + *indicates chromatophore present*).

	Antennata[1]	Kempi	Paucibranchiata	Pulchra[1,2]
Number of eyes (12 to 13 setigers)	6	4	6	4
Pigment				
Dorsal median pigment spots				
Anterior	+	–	+	+
Pygidium	+	+ (small)	+	+
Number dorsal rows	2	4	2	4
Anterior dorsal pigment pattern				
Prostomium	–	–	–	+
Setiger 1	+	–	+	++++
Setiger 2	–	–	+ +	++++
Setiger 3	–	++++	+ +	++++
Setiger 4	+ +	++++	+ +	++++
Setiger 5	+ +	++++	+ +	++++
Ventral segmental pigment pattern	++ (between setigers 1–2 only)	++ (setigers 2–10)	+ (setiger 6)	–
Reflective yellow	absent	absent	present	present
Dorsal dispersed green	absent	absent	present	present
Palp pigment	absent	spots numerous irregular white	spots regular reflective yellow	bands black and green
Approximate size: (13 setiger stage)	860 μ	750 μ	640 μ	1450 μ

[1] Data from Hannerz (1956).
[2] Data from Rullier (1963).

tinue feeding on nurse cells until all are eaten; then, the larvae leave the capsule. A similar pattern occurs in *Polydora hoplura* Claparède (Wilson 1928), *P. nuchalis* (Woodwick, 1960) and *Boccardia proboscidea* (Hartman, 1940, 1941; Woodwick, unpublished).

The morphology of *Pseudopolydora paucibranchiata* and of *P. kempi* larvae can be compared with that of *P. antennata* larvae described by Hannerz (1956) and *Pseudopolydora pulchra* larvae described by Casanova (1952), Rullier (1963) and Hannerz (1956). Pigmentation appears to be the most useful characteristic and is emphasized in Table II, although no groupings or special relationships of species are evident from the pigmentation patterns delineated. One striking alternative characteristic is the comparatively large size of *P. pulchra* larvae.

The characteristics noted in Table II for larvae of *P. antennata* and *P. pulchra* were taken from Hannerz (1956). It is of interest to note, however that there is apparent variation in the pigment among the published descriptions of *P. pulchra* larvae.

California adult specimens of *P. kempi* collected by us agree with descriptions given by Okuda (1937) for the same species in Japan. They have a well-developed nuchal tentacle and the peculiar pygidium with two dorsal lappets. These observations are based on specimens collected from Morro Bay, San Francisco Bay, Bolinas Lagoon, Tomales Bay and Bodega Harbor. The specimens collected by Light (1969), however, were said to lack a nuchal tentacle and to have a different pygidial structure.

In order to shed some light on this apparent anomaly, one of us (JAB) examined the type material of *P. kempi californica* deposited in the California Academy of Sciences. The type material consists of the holotype which is badly preserved and two paratypes, each of which is fragmented and incomplete. All three specimens lacked the nuchal tentacle. The second paratype, however, had a small circular scar just posterior to the second pair of eyes which suggests that the nuchal tentacle was broken off. The first paratype was damaged in the same area and an irregular slit marked the possible site of a nuchal tentacle. A pygidium was present only on the holotype, but was so badly preserved and contracted that its true structure was impossible to determine.

Based on our observations of living *P. kempi* adults and juveniles and upon examination of the type collection of *P. kempi californica* Light we conclude that California specimens agree well enough with descriptions from Japan to preclude the necessity of the subspecies name *californica*. This conclusion is further supported by Carlton (1975) who suggests that *P. kempi, P. paucibranchiata* and many other California marine invertebrates may have been introduced from Japan.

SUMMARY

1. The larval development of *Pseudopolydora paucibranchiata* (Okuda) and *P. kempi* (Southern) is described. Both species occur in tidal flats of California bays and estuaries.

2. Adult females of *P. paucibranchiata* deposit eggs in capsules which are attached to the inner lining of their tubes. All eggs are fertilized. Larvae develop in the capsules until they have 3 setigerous segments at which time they enter the plankton. After development of 13–17 setigers they begin to settle out of the plankton and assume a benthic life.

3. Eggs of *P. kempi* are also deposited in capsules, but in this case only a small percentage are fertilized. The unfertilized eggs fragment into separate yolk granules and are eaten by the developing embryos. After all yolk is devoured the larvae continue their development sustained by this stored food reserve. They remain in the capsule until they have about 15 setigers. They remain in the plankton only a short time before settling and taking up a benthic life.

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SIDEREAL-DAY VARIATION IN SPONTANEOUS ACTIVITY OF THE MOUSE, *MUS MUSCULUS* [1]

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A body of data has been developed to indicate that the spontaneous motor activity of a number of small mammals is not random in its variation but tends to possess recurring patterns. These patterns include solar-day, lunar-day, synodic-monthly, and annual periodicities (Boyer, 1970; Brown, F. A., 1965; Brown and Park, 1967; Brown, Shriner, and Ralph, 1956; Brown, J. A., 1973; DeCoursey, 1959; Johnson, 1939; Kavanau, 1962, 1969; Stutz, 1970, 1972, 1973, 1974; Terracini and Brown, 1961, 1962).

In a study of the spontaneous motor activity of the mouse, *Mus musculus*, a rhythm of even another periodicity has been observed (Brown, J. A., 1973). This is the sidereal-day pattern of mean activity. Due to the great distance of the stars from the earth, rotation relative to them constitutes the earth's actual period of the rotation. This is the sidereal-day and has a period of 23 hours 56 minutes. The sidereal-day is close to four minutes shorter than the solar-day cycle (24 hours). The sideral-day, therefore, as a consequence of the annual orbital passage of the rotating earth around the sun gains on the solar-day two hours each month or just 24 hours in a year. A rhythm of this period has also been observed in the pattern of oxygen consumption in potatoes (Brown, F. A., 1958).

This paper will present the characteristics of this observed sidereal-day pattern and will discuss the possible relation of this pattern to the timing of annual rhythms and nocturnal navigation.

Materials and Methods

Twelve adult male Swiss white mice, *Mus musculus*, three months old at the beginning of the experiment were used. Each mouse was maintained in the experiment until sickness or death resulted. At this time another mouse was substituted as the experiment continued. The experiment ran continuously from June 1, 1965 to May 31, 1966.

Each mouse was housed in a tilting-cage actograph similar to the type used by Terracini and Brown (1962). The rocking cage movements effected by the movement of the animal were transformed by the recording system, an Esterline Angus events recorder, into one lateral movement of a recording pen for each excursion of the mouse to an opposite side of the "track". The rate of movement of the paper was 0.75 inches an hour or 18 inches a day. The movements of the mouse from one side of the cage to the other involved, obviously, running or walking activity.

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[1] This research was based upon a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree in biological sciences at Northwestern University, Evanston, Illinois, in June 1973.

The mice were subjected to the natural variation in the duration of illumination. The temperature of the room was maintained at approximately 22° C, thermostatically controlled throughout the colder months of the year, but rising during warmer days of summer to 25° C or higher. Water and food were constantly available to the mice. The times of renewing water and food were varied randomly as were all disturbances associated with servicing the actographs and recording systems.

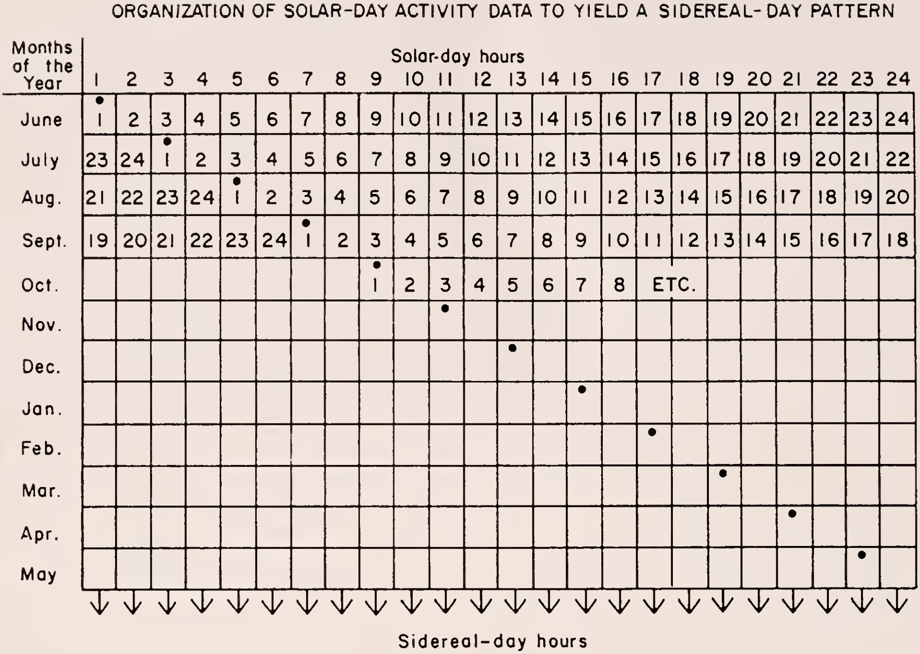
The data which were used were obtained from the actograph recordings and quantified in the following manner: (1) the hour by hour (all times were Central Standard) activity records for each mouse, individually, were given numerical values in terms of a scale ranging from 0 to 10. The numbers described the fractions of the hour that the animal was active. For example, 0 indicated no activity, 2 indicated 20% of the hour, and 10 was 100% of the hour. (2) These values were then tabulated to indicate the hourly numerical units of activity for each mouse for each day of the experiment. (3) The individual hourly data for all mice were then combined into monthly tables showing the combined total activity for all the mice for each hour of each day of the consecutive months. These monthly tables served as the basis for further analysis of the sidereal variational patterns of the spontaneous motor activity of the mice.

The pattern of the mean sidereal cycle can be obtained from units of a year of hourly data by simply displacing consecutive mean monthly 24-hour cycles to progessively two-hour later relationships in the day. Using data for a whole year will obviously randomize the mean solar-day and mean lunar-day and, for all practical purposes, the monthly components as well. These mean hourly values so aligned in columns of the twelve monthly rows of mean 24-hour data are essentially the hours (± 1) of the sidereal-day (see Figure 1).

Since sidereal midnight is defined as the instant at which the vernal equinox crosses the upper meridian, and sidereal noon the instant the vernal equinox crosses the lower meridian, sidereal hour 24 (0) will lie within that vertical column containing solar-day noon for March–April and solar-day midnight for September–October. Because for the originally monitored solar-day data the 12th hour represented the amount of spontaneous motor activity from 11 to 12 AM and the 13th hour represented activity from 12 AM to 1 PM, and also because of the 2-hour slide, the data were combined into 2-hour periods in the solar-day. The two-hour means can thus come close to being centered on the sidereal times of noon and midnight. While sidereal 0 and 12 reach upper transit at solar-day noon on the vernal and autumnal equinoxes, respectively, the 6th hour of the sidereal-day reaches upper transit at noon on the summer solstice about June 21. Comparably, the 18th sidereal hour reaches upper transit at noon on the winter solstice about December 22.

RESULTS

The sidereal-day pattern obtained from the data on the spontaneous motor activity of the mice studied from June 1, 1965 through May 31, 1966 is indicated in Figure 2. The approximate hours of the sidereal-day are indicated on the abscissa. The mean hourly value for the year is 18.8. The mean hourly values, expressed as deviation from the yearly mean, range from − 4.7 to + 3.8. The



ORGANIZATION OF SOLAR-DAY ACTIVITY DATA TO YIELD A SIDEREAL-DAY PATTERN

Months of the Year	Solar-day hours 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
June	● 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
July	23	24	● 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Aug.	21	22	23	24	● 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Sept.	19	20	21	22	23	24	● 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Oct.									● 1	2	3	4	5	6	7	8	ETC.							
Nov.											●													
Dec.													●											
Jan.															●									
Feb.																	●							
Mar.																			●					
Apr.																					●			
May																							●	

FIGURE 1. The values within the boxes indicate the hourly organization as they are shifted two hours later each month.

range of variation is 40% of the overall mean value. The gradual shift of the phase relationship of the sidereal cycle to the daily cycle of the mice is diagrammatically depicted in Figure 3.

The sidereal pattern for the mouse data comprises a unimodal variation with a maximum occurring at the midnight hour (0) of the sidereal day and a minimum at the 12th hour. There has been to date only a single search in organisms for a variation correlated with the sidereal period. A mean pattern of variation possessing this period was reported for O_2-consumption in potatoes for a two-year study (Brown, F. A., 1958). The sidereal pattern of the mouse differs from the sidereal component of O_2-consumption in potatoes observed over the period from February 1, 1956 to January 31, 1958 in being about 8 hours, or 120°, phase-advanced. In general form they appear to be moderately similar. An eight-hour lead correlation of the mouse cycle on the potato yielded $r = +0.86 \pm 0.08$. The phase difference between the mouse and the potato cycles was not the result of a difference in the years at which the investigations were conducted because further continuation of the potato study through 1967 (including the interval of the mice study) discloses no difference in phase or form of the cycle as compared with the earlier sidereal analysis. Results obtained from an 11-year sidereal analysis for the potato (Figure 4) were obtained through personal communication with Dr. F. A. Brown, Jr., Northwestern University (see also Brown, F. A., 1973).

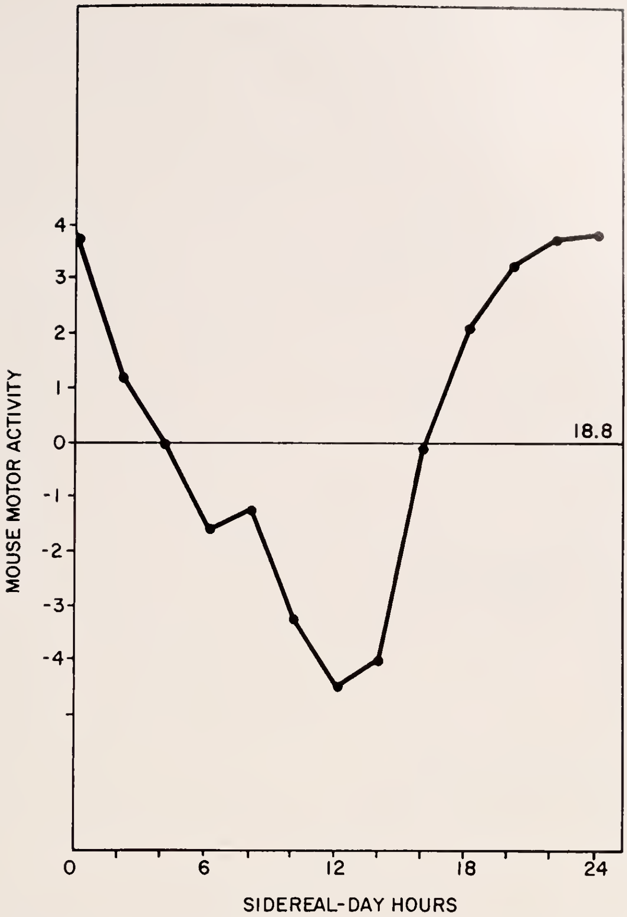


FIGURE 2. Values are two-hour averages expressed as deviation from the mean hourly sidereal-day activity, indicated on the abscissa.

DISCUSSION

The unimodal sidereal-day cycle of spontaneous mouse motor activity represents another example of a behavioral pattern synchronized with a major geophysical cycle. Other evidence of geophysically correlated periodisms in the mouse, *Mus musculus,* is reported in Boyer, 1970; Brown, J. A., 1973; Terracini and Brown, 1962; Truchan and Boyer, 1972. Variations of sidereal-day periods have been observed for the electromagentic fields of the earth. The sensitivity of organisms to weak electromagnetic fields of the intensity as found on earth has been well documented (Brown, F. A., 1971; Brown, Park, and Zeno, 1966; Lindauer and Martin, 1968; Rommel and McCleave, 1972; Wiltschko and Wiltschko, 1972; and many others). The occurrence of another geophysically correlated pattern, the sidereal-day one, seems to add more evidence to the recent studies

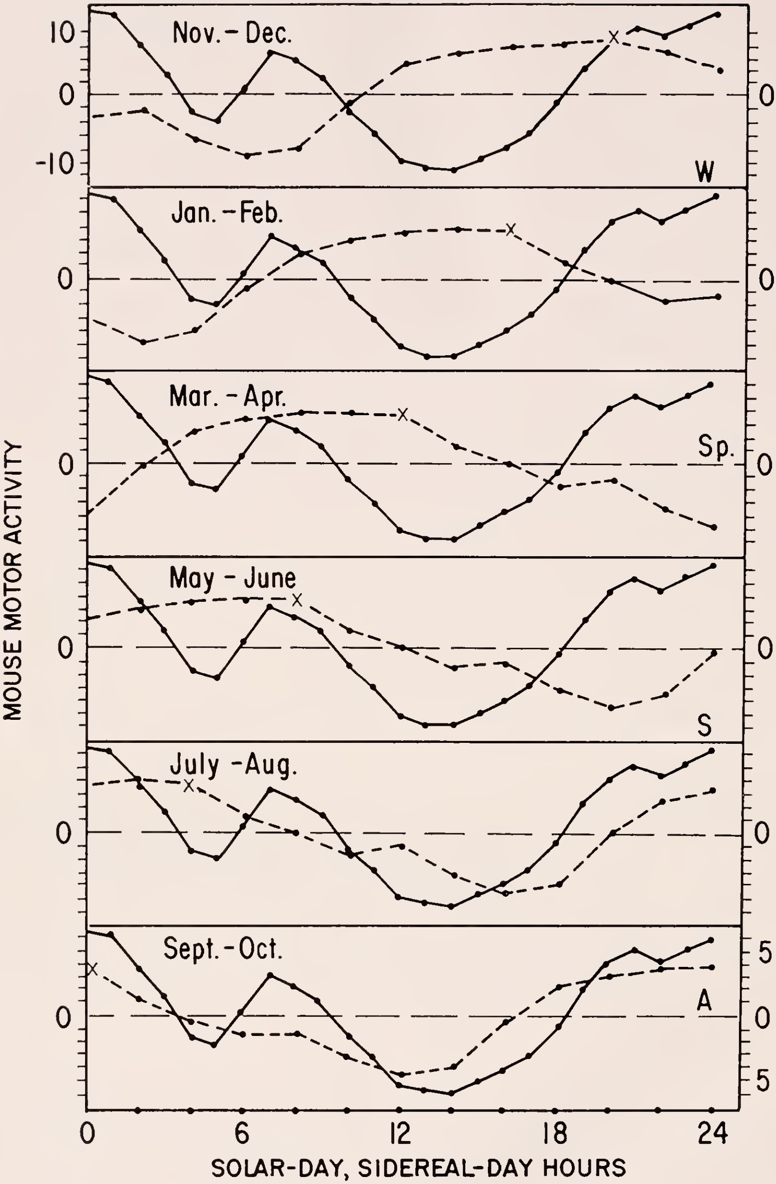


FIGURE 3. The yearly movement of the sidereal-day activity pattern across the mean solar-day activity pattern.

Mean solar-day pattern is indicated by the solid line. Sidereal-day pattern is indicated by the dashed line.

All the values are expressed as direction and deviation from a common mean indicated by the dashed line. Solar-day values are indicated on the left ordinate. Sidereal-day values are indicated on the right ordinate.

The x describes the movement of the (O) sidereal hour as it crosses the solar-day pattern during the year. W indicates the time of winter solstice; Sp.—the spring equinox, S—the summer solstice, and A—the autumnal equinox.

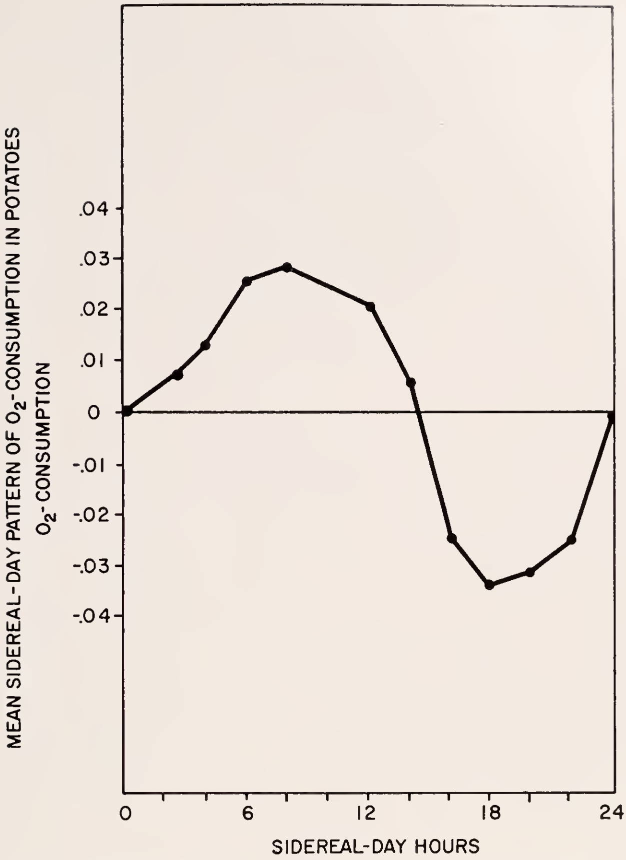


FIGURE 4. Potatoes were maintained in constant conditions of all obvious factors February 1, 1956 through January 31, 1967. Cycle range is about 1 percent of the mean rate.

that indicate these weak electromagnetic fields may be the timing mechanism for the biological clock (Brown, F. A., 1972; Brown, and Chow, 1973).

A mean annual pattern of spontaneous motor activity in the mouse, *Mus musculus,* has been observed (Brown, J. A., 1973). An annual rhythm can result from the periodic reinforcement of the solar-day cycle by the sidereal one of 23 hours and 56 minutes. The sidereal-day cycle scans across the 24 hour day in exactly one year (Figure 3). As another example of a timing mechanism that employs the changing phase-relationships between two basic geophysically related cycles to yield a third there has been observed the mean pattern of synodic-monthly frequency (29.53 days). This periodism is the result, in at least some measure, of the systematically altering phase-relationships between the 24-hour solar-day and the 24.8-hour lunar-day, with consequent periodic reinforcement of the solar-day maxima with a mean synodic-monthly frequency (29.53 days). The presence of a pattern possessing this period in the spontaneous activity pattern of the mouse has been noted previously by Boyer (1970), J. A. Brown (1973), and Terracini and Brown (1962). The existence of this periodicity in other mammals has been observed for hamsters (Brown and Park, 1967), rats (Brown, Shriner, and Ralph, 1956) and Mongolian gerbils (Stutz, 1973).

One more role that the presence of a sidereal-day pattern might play in the behavior of animals is involved with the mechanisms used by animals for nocturnal navigation. Although there seems to be little total agreement as to the mechanism that is used by birds specifically in nocturnal navigation, if the use of celestial bodies as navigational reference points is to be considered reasonable, the ability of birds to compensate for the apparent motion of the stars necessitates a biological clock with a period of the sidereal-day rhythm (Wallace, 1973). We have mounting evidence in both potatoes and mice that at least certain organisms do possess patterns of this period length. Further study to indicate such a recurring pattern in other organisms is necessary.

Summary

1. Spontaneous motor activity of twelve adult male Swiss white mice was monitored for the year June 1, 1965 through May 31, 1966, in Evanston, Illinois. The mice were maintained in natural illumination in the laboratory.

2. A mean sidereal-day activity pattern was disclosed. This comprised a unimodal variation with a maximum occurring at sidereal midnight (hour 0) and a minimum at the 12th hour. The range of the cycle was 40% of the mean. It is postulated that the sidereal variational pattern reflects biological responsiveness to the mean sidereal-day fluctuations in the geoelectromagnetic field.

3. The presence of an annual pattern of spontaneous motor activity is postulated to be the result, in at least some measure, of the systematically altering phase-relationships between the 24-hour solar-day and the 23-hour 56-minutes sidereal-day as the sidereal-day makes it passage across the solar-day to be completed exactly in one year's period.

4. The significance of a sidereal-day periodicity to nocturnally migrating organisms is postulated.

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BIOMECHANICS OF WATER-PUMPING BY *CHAETOPTERUS VARIOPEDATUS* RENIER. SKELETOMUSCULATURE AND KINEMATICS

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The tube-dwelling polychaete, *Chaetopterus variopedatus*, has long been recognized as being the most structurally specialized of all annelids. Although reasonably detailed and accurate accounts of the basic anatomy of *Chaetopterus* appeared near the turn of the century (Joyeux-Laffuie, 1890; Enders, 1909), the functional correlates of certain of the structural peculiarities have not been examined in detail. One of the most obvious gaps in our knowledge concerns the mechanism of water-pumping. *Chaetopterus,* like other aquatic animals which live totally confined within tube-houses or burrow systems, must actively irrigate its dwelling to provide an oxygen supply adequate to meet its respiratory demands. In addition, it has been shown that the filter-feeding mechanism of *Chaetopterus* is completely dependent upon the water-pumping activities of the animal (MacGinitie, 1939). It was early recognized that the three disk-shaped segments (numbers 14, 15, and 16) are the agents responsible for water propulsion, but the skeletomusculature of these particular segments has never been described in detail. Although a number of workers have considered different aspects of water propulsion by *Chaetopterus* (Berrill, 1927, 1928; Wells and Dales, 1961; Barnes, 1964, 1965), none has directly examined the mechanism involved. However, there appears to be general agreement that the behavior of the pump segments does not involve a simple peristaltic or undulatory motion—the two most common methods used by annelids to propel water through tubes. The present investigation was undertaken, therefore, to examine in detail the mechanism of water propulsion by *Chaetopterus.* This paper presents a description of the skeletomusculature and an analysis of the kinematics of the pump segments.

MATERIALS AND METHODS

Source and maintenance of animals

Specimens of *Chaetopterus* were obtained from Pacific Bio-marine Supply Co., Venice, California. In the laboratory, the animals, within their natural tubes, were maintained at 16° C in aerated recirculating aquaria containing 50 gallons of artificial seawater ("Instant Ocean", Aquarium Systems, Inc., Cleveland, Ohio). The worms were fed *ad libitum* daily with "Fryfare" extra-fine fish food.

Observational techniques

Since the animals could not be viewed directly in their natural tubes (due to the opacity of the tube wall), the worms were placed in glass tubes of equivalent

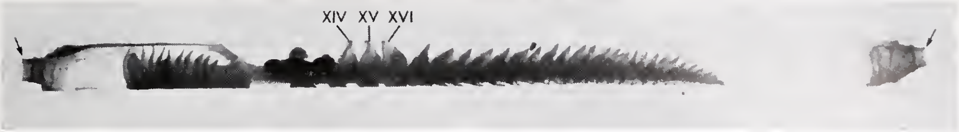


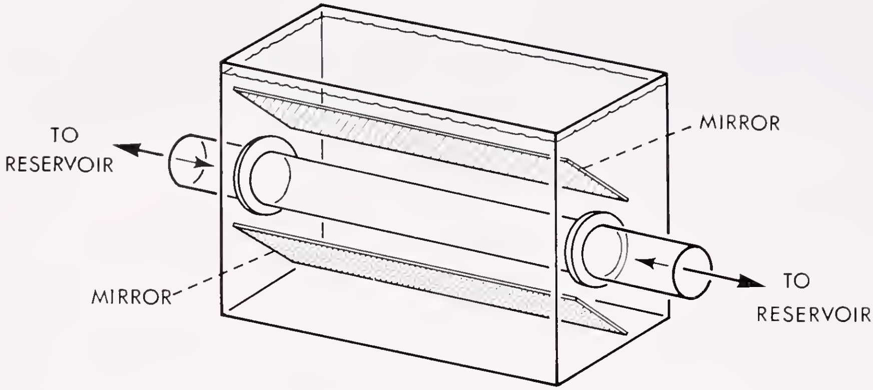
FIGURE 1. *Chaetopterus* in a glass observation tube which has been lined with the natural tube material. Terminal constrictions indicated by arrows; roman numerals correspond to the three pumping segments.

diameter prior to observation. Within 2 days after transfer, they had lined the glass tubes with a thin, transparent film of their natural tube material and had constructed the characteristic terminal constrictions at the ends of the tubes (Fig. 1). The environment immediately surrounding the worms, therefore, was nearly identical to that of the natural situation. Two lines of evidence, moreover, suggest that the pumping behavior of *Chaetopterus* in the observation tubes was well within the normal limits of behavior patterns of the worms in nature. First, pumping activity, as measured by the "surge-chamber" method of Wells and Dales (1951), or by pressure transducers, yielded identical data for worms in natural tubes and worms in lined glass tubes. Secondly, individual specimens of *Chaetopterus* have been maintained in a healthy condition in such lined glass tubes for over six months in our aquaria, indicating that the feeding mechanism (which is completely dependent upon pumping activity) is unimpaired. It would appear unlikely, therefore, that the pumping behavior of the worms, at least at the biomechanical level, was adversely affected by the substitution of glass tubes for the natural ones.

Prior to filming, a glass tube containing an active, acclimatized worm was placed in the viewing chamber shown in Figures 2 and 3. This chamber was made of $\frac{3}{16}$ inch plexiglas on the sides and bottom, with $\frac{1}{16}$ inch sheet glass for front and rear walls. Two front-surfaced mirrors inclined at 45° angles, together with appropriate illumination and masking, allowed simultaneous filming of top and side views of an actively pumping worm. Interconnected two-liter reservoirs at each end of the glass tube permitted the worm to pump water in either direction without development of an asymmetric pressure head. These reservoirs likewise served as sites where oxygenation and food addition could be accomplished without disturbing the animal. All observations were made at water temperatures of 16° C. Local heating effects during filming were minimized by interposing a 4 inch thick water-filled heat absorber between the light source and the viewing chamber. In addition, the viewing chamber itself was water-filled, thereby improving the optics of the system as well as acting as a second heat-absorber. Filming was done with a motor-driven Bolex 16 mm movie camera (at 16 and 24 f.p.s.) or with a motor-driven Nikon 35 mm single-lens reflex (at 4 f.p.s.). Kodak Plus-X negative film was used in either case. Frame-by-frame analysis of the 16 mm film was carried out with the aid of a L.W. Photo-optical Data Analyzer.

Anatomical methods

For detailed examination of the musculature, specimens were fixed by injection/immersion in cacodylate-buffered 6.5% glutaraldehyde (pH 7.2) and subsequently



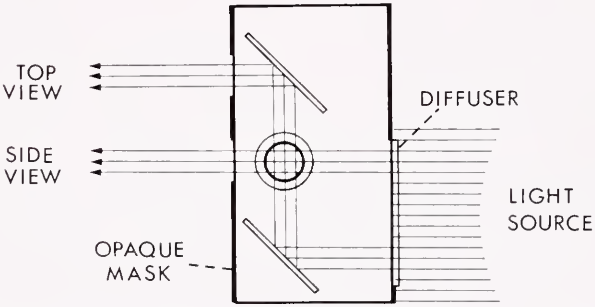


FIGURE 2. Observation chamber; see text for details of construction.
FIGURE 3. End view of observation chamber showing optics of system.

differentiated in 1% osmium tetroxide. Dissections were performed with the aid of a Bausch and Lomb stereomicroscope.

RESULTS

Skeletomusculature

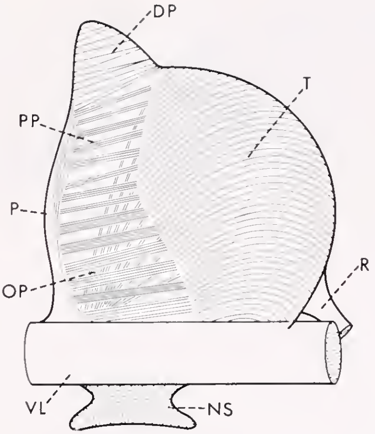
As in all annelids, the functional skeleton of *Chaetopterus* is hydrostatic. Coelomic fluid occupies an estimated 85% of the volume within each piston segment, with intestine, nephridia, and gametes making up the balance. There is good anatomical evidence (Joyeux-Laffuie, 1890; Enders, 1909; Meissner, 1935) that the coelomic fluid contained within each piston segment is completely isolated, and my own experience with dye and fixative injection, or fluid removal, is consistent with this view. Likewise, there were no indications during the present study that the volumes of the piston segments varied during pumping activity.

The general body wall of *Chaetopterus* is known to be unusually thin and elastic for a polychaete of its size (Storch and Welsch, 1970; Brown, Bdzil, and Frisch, 1972), and this is especially true for the integument of the pumping segments. Previous studies indicate that, with regard to external morphology and developmental homology (Joyeux-Laffuie, 1890; Enders, 1909), coelomic cavity and gonoduct structure (Meissner, 1935), and nervous system structural organization (Berrill, 1927), the three pump segments can be considered equivalent. The present investigator likewise found no differences in the muscular organization, and, accordingly, the description that follows applies equally well to each of the three pump segments.

Figure 4 shows the musculature visible within the right half of a piston segment, following removal of the intestine and nephridium. The so-called neuropodial "sucker" forms a base across which lies the large right ventral longitudinal muscle. Along the inner face of the posterior wall the thin transverse muscle fibers run in parallel courses. On the anterior wall the right promotor muscle ascends from its origin on the ventral longitudinal muscle and fans out dorsally, ultimately to terminate at multiple insertion points. Looking deeply into the contiguous parapodial cavity, one can see three distinct sets of muscles running across the cavity between anterior and posterior walls. These include: numerous fine distal parapodial fibers, which occupy the greatest area of the parapodial margin; more robust proximal parapodial muscle bands, which incompletely divide the parapodial cavity from the main chamber; and oblique parapodial muscle bands, which originate together anteriorly at the ventral longitudinal muscle, radiate in a dorsoventral plane, and ultimately terminate at numerous insertion points along the posterior wall. The relationship of distal and proximal parapodial muscles can be further seen in Figure 5, which shows a frontal section taken approximately midway along the dorsoventral axis. The anterior wall, as viewed from the interior, appears as shown in Figure 6. The large promotor muscles originate ventrally and terminate centrally at the midline, and laterally at, or near, the points of insertion of the proximal parapodial muscle bands. In the central region are also found numerous closely-spaced anterior radial (I) fibers. At the margin of the front wall are located peripheral anterior radial (II) fibers and submarginal fibers running parallel to the edge (anterior circular fibers). The musculature of the posterior wall of a piston segment is shown in Figure 7, as seen from the outside. The central region is dominated by a complex web of decussating fibers arising from left and right remotor bundles. Remarkably, these remotor bundles originate within the musculature of the ventral neuropodial "sucker" of the *adjacent* posterior segment, and thus are clearly *intersegmental* in nature. Circular and radial muscle fibers occupy the margin of the posterior wall, with their relative position being reversed from that found in the anterior wall. As previously shown (Fig. 4), the inner surface of the posterior wall contains a sheet of parallel transverse fibers.

Posture, orientation, and position during water-pumping

During most periods of active pumping, the worms are extended to approximately 1.5 times their fully-contracted length. The major contribution to this extended posture occurs in segment 13 (bearing the cupula), and to a lesser, ④



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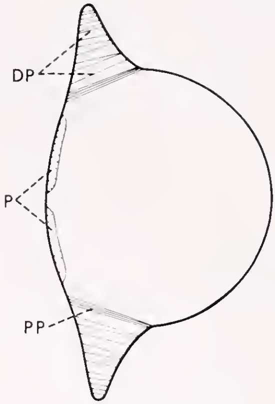

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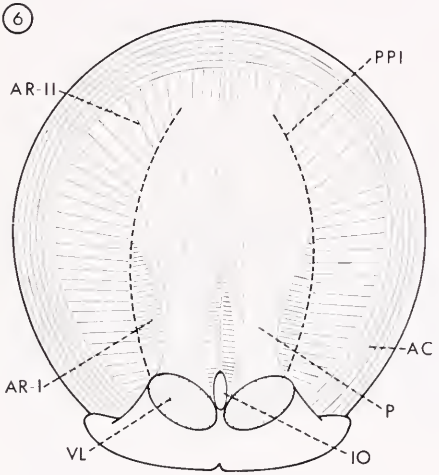

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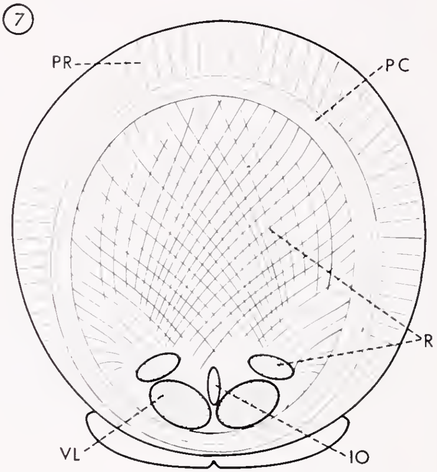


Figure 4. Musculature within right half of a pump segment; DP, distal parapodial; PP, proximal parapodial; OP, oblique parapodial; P, promotor; VL, ventral longitudinal; NS, neuropodial "sucker"; R, remotor; T, transverse.

Figure 5. Musculature of a pump segment as seen in frontal section; PP, proximal parapodial; P, promotor; DP, distal parapodial.

Figure 6. Musculature of the anterior wall of a pump segment; PPI, proximal parapodial insertions; AC, anterior circular; P, promotor; IO, intestinal opening; VL, ventral longitudinal; AR-I, AR-II, central and peripheral anterior radial.

Figure 7. Musculature of the posterior wall of a pump segment; PC, posterior circular; R, remotor; IO, intestinal opening; VL, ventral longitudinal; PR, posterior radial.

but significant, extent by the water-propelling segments (14–16). Full extension is achieved by slow, forward locomotion of the anterior part of the worm (segments 1–12), while the posterior part of the animal (segments 17 and beyond) remains stationary. Within the observation tubes, the preferred orientation was ventral side down, the entire length of the animal. The most common postural variant was that in which the anterior segments (1–12) skewed in either direction about the longitudinal axis, segment 13 would be twisted, and segments 14 and beyond would remain unaffected. Occasionally a worm would take up an inverted orientation (clinging to the roof of the tube), with or without a skewness in posture. Since all of the observation tubes were at least 5–10 cm longer than the worms, the animals had ample opportunity to move linearly within their tubes. The worms showed a decided preference for positioning themselves towards the temporary incurrent end, but neither opening was consistently chosen for this functional role. Although neither skewness nor inverted orientation appeared to alter pumping activity to any significant degree, all of the measurements reported here were taken from animals in the extended, axially-aligned posture and in the ventral-side-down orientation.

General characteristics of pumping activity

Figure 8 shows film records of the sequence of activities which occurs during one complete pump cycle. The most obvious component of the cycle is the axial displacement of the dorsal and lateral portions of segments 14–16. Close observation of individual piston segments further reveals that well-defined radial movements occur at specific intervals during axial displacement. During the backward (power) stroke, the margin of each pump segment is extended to meet the inner face of the tube wall; during the forward (recovery) stroke, the segmental margin is retracted, leaving a clearance, dorsally and laterally, between worm and tube wall. There exists a high degree of both intrasegmental and intersegmental coordination and it appears that segments other than numbers 14, 15, and 16 remain virtually motionless during pumping activity.

Displacements and shape changes

Eleven worms were filmed during active water-propulsion and the simultaneous axial and radial displacements of the dorsal and lateral margins of the piston segments were determined. To compensate for absolute size differences and rates of pumping, the data were normalized and then subjected to direct graphical comparison. Composite results are shown in Figures 9 and 10. These curves should be taken as "modes", rather than true arithmetic "means" of all of the worms measured. The time and distance marks are likewise indicative of mode values for worms weighing 6–9 gms, in aerated seawater at 16° C. In the clear majority of cases, the axial displacements (Fig. 9) approximated a pure sine function. In any single worm there were no detectable performance differences among the three piston segments. The only variation between worms which appeared more than once in the records occurred during the recovery phase, when the segments failed to initiate recovery immediately upon reaching their most posterior position. However, since the velocity of the subsequent recovery movement was always increased, the relative duration of the total recovery stroke remained at 50% of the complete cycle. All power phase records were virtually identical. Calculated velocity and acceleration curves, together with representative absolute values, are also given.

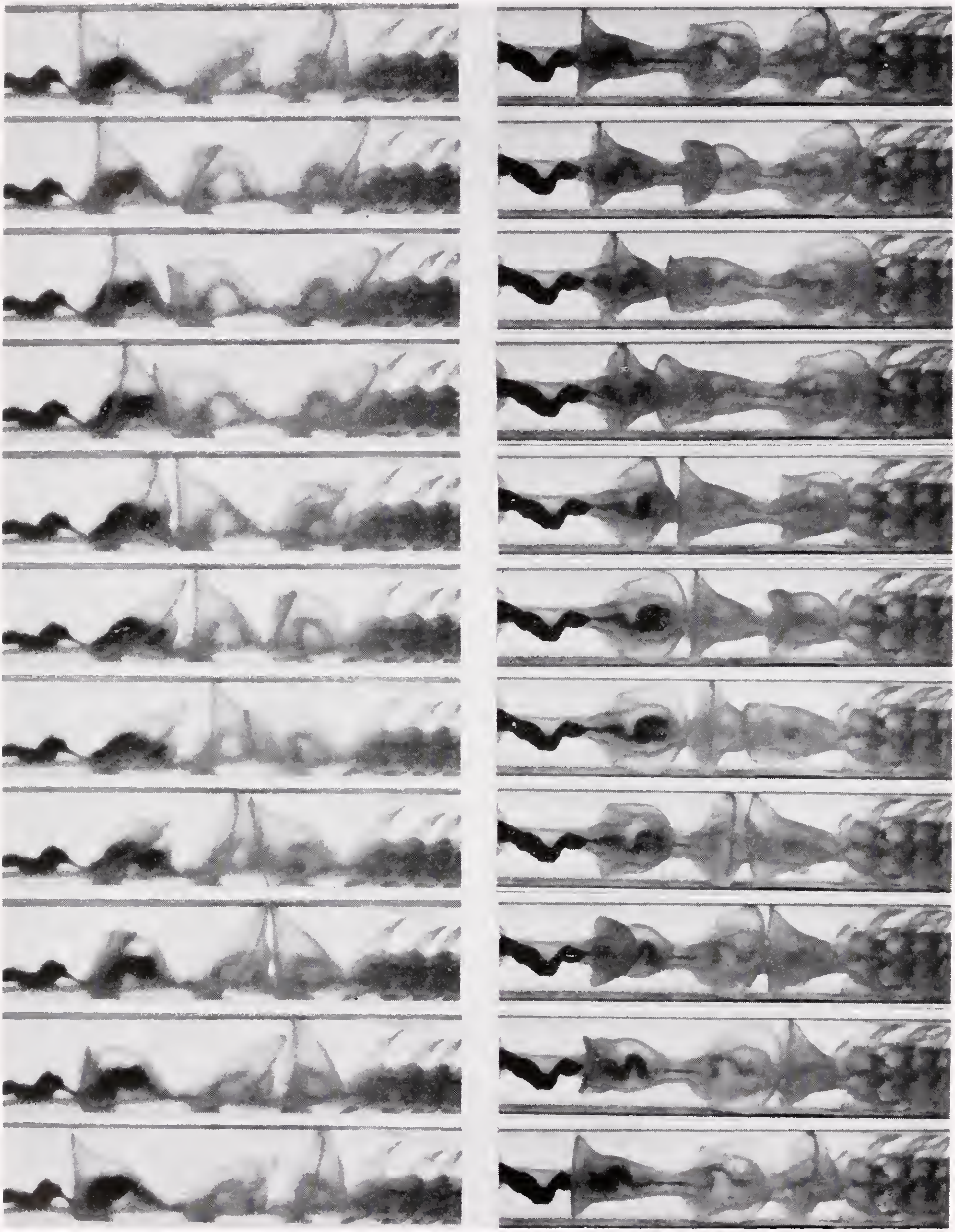


FIGURE 8. Sequence of activities during one complete pump cycle in *Chaetopterus* (anterior of worm to left); left-hand column as seen from side, right-hand column as seen simultaneously from top. Time between exposures is approximately 0.25 sec.

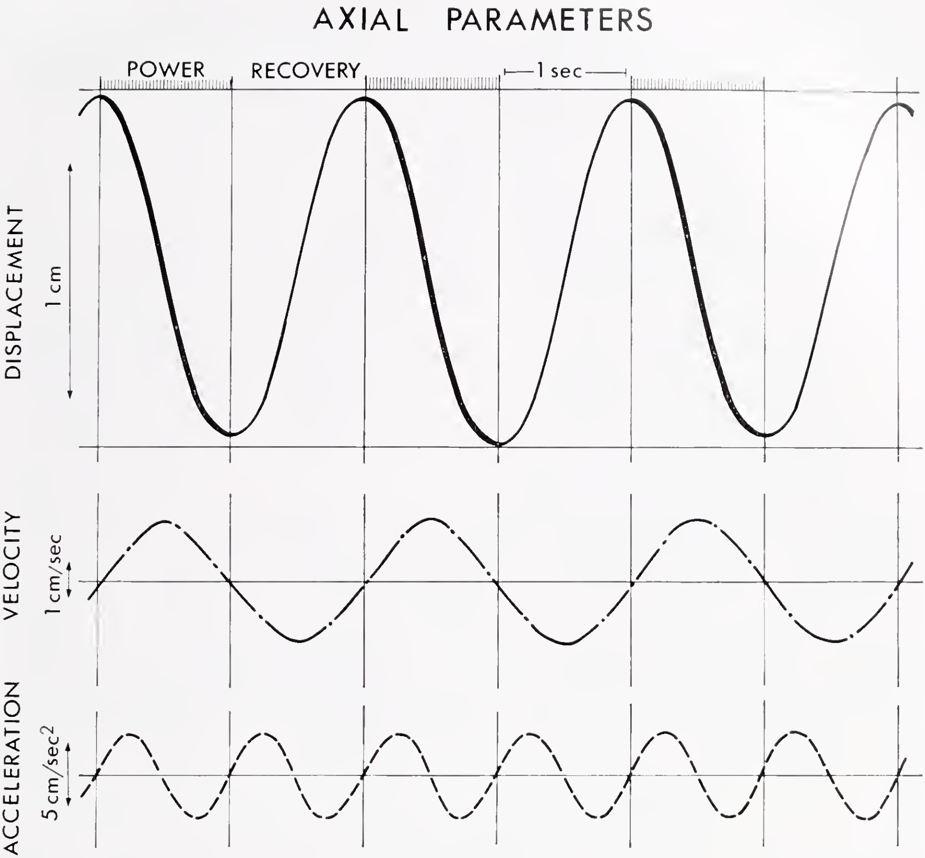


FIGURE 9. Characteristic maximum axial displacements of the pump segment periphery during 3 consecutive pump cycles.

Modal characteristics for radial displacements are shown in Figure 10. The displacements here are clearly asymmetric, with the centrifugal movement limited by the inner margin of the tube wall. Although the total distances moved radially by the margins of the piston segments are less than ¼ of the distance moved axially, the radial movements are at least twice as rapid as the axial—as seen in the derived velocity/acceleration curves. Simultaneous plots of the axial and radial displacements yield the trajectories shown in Figure 11A. In order to evaluate the changes in shape which occur, selected frames from one complete pump cycle were projected and drawn in superimposed positions. Since no significant differences among the segments could be detected, the shape changes for only one segment are given in Figures 11B and 11C, for clarity. These latter figures clearly indicate the shape changes to be great, in spite of the fact that at all times during the pump cycle the piston segments remain nearly circular in cross-section. During the power phase, the mobile portion of a piston segment assumes a geometry which loosely approximates a biconvex disk. The inner wall of the

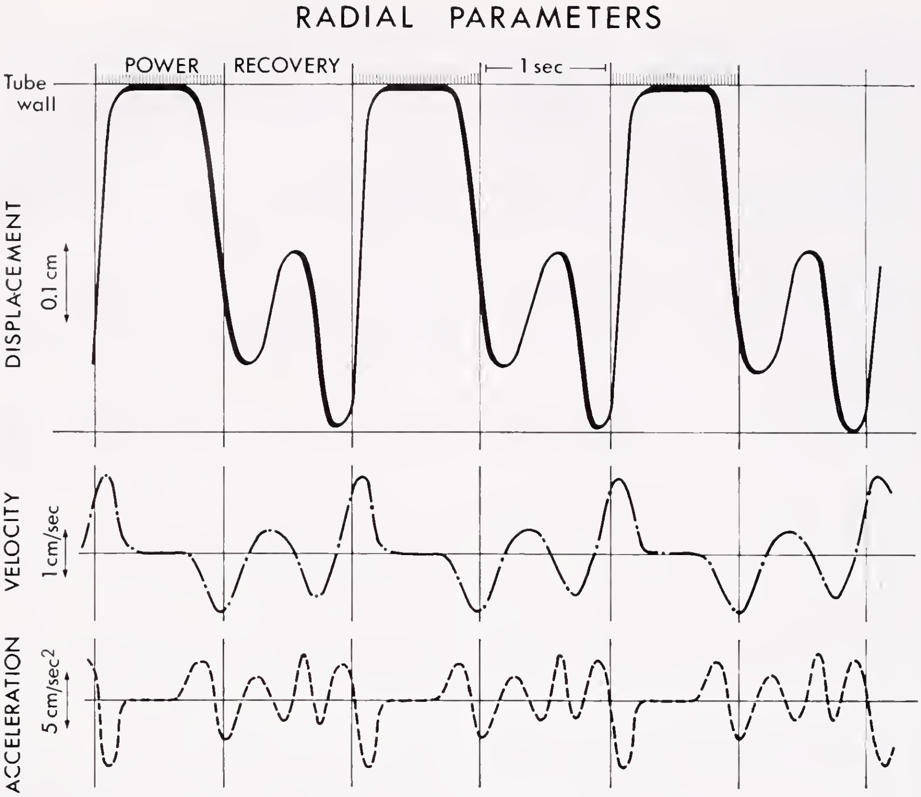


FIGURE 10. Characteristic maximum radial displacements of the pump segment periphery during 3 consecutive pump cycles.

tube sets a maximum limit on the amount of radial extension which can occur. During the initial part of the recovery stroke, the mobile portion of a piston segment rapidly assumes a nearly spherical shape. The minimum diameter of this sphere is undoubtedly set by the volume of incompressible coelomic fluid contained within the segment. Recovery is continued by a forward rocking/rolling motion, and the final transition from sphere to biconvex disk is rapidly accomplished at the climax of the recovery stroke.

Muscle action during the pump cycle

Although it was not feasible to record directly the sequence and duration of muscle contraction using standard myographic techniques, two features of the system made it possible to analyze the muscle activities with reasonable confidence of accuracy. The first of these was the fact that although the total musculature of a piston segment is complex, the courses of individul sets of muscles are relatively simple and straightforward (the remotors being an obvious, but manageable, exception). Secondly, the ability to record simultaneous top and side views of the

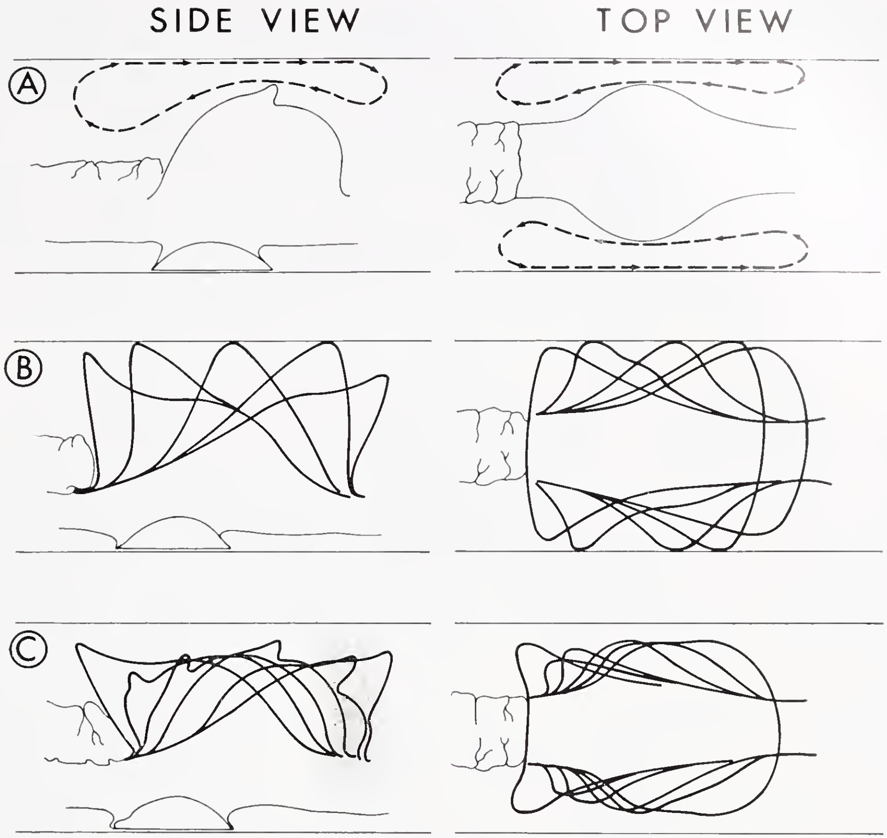
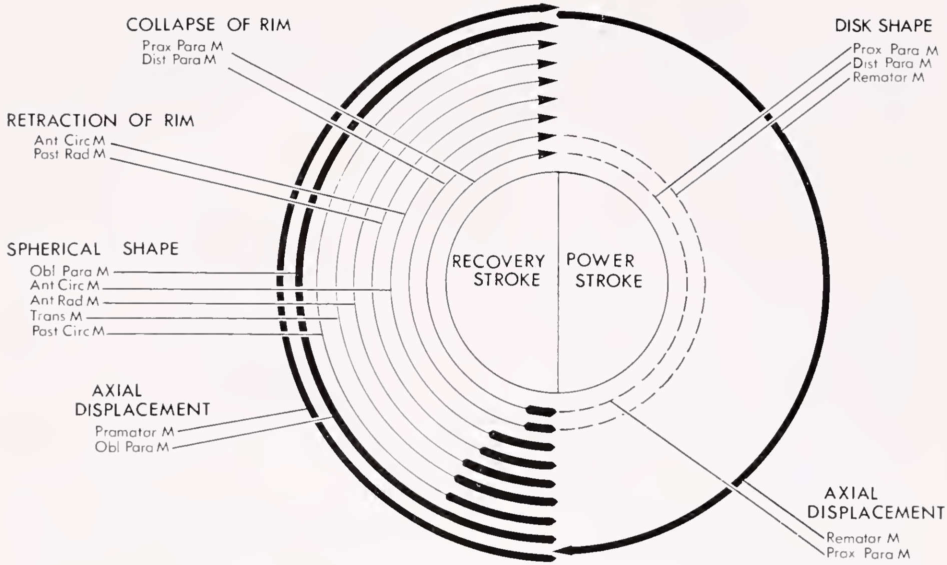


FIGURE 11. (A) Trajectory (side and top view) of margin of pump segments during a complete pump cycle; (B) shape changes (side and top view) during the power stroke; (C) shape changes (side and top view) during the recovery stroke.

relatively slow shape changes (*ca.* 2+ seconds for a complete cycle) at relatively fast film speeds (24 f.p.s.) enabled the onset of even small absolute displacements (*e.g.*, in the radial direction) to be easily detected and measured. A diagram summarizing the deduced sequence, duration, and action of the various muscle groups during a single complete pump cycle is presented in Figure 12.

Starting at the 12:00 o'clock position on the diagram and proceeding clockwise, I will first consider the power stroke. Careful examination of the films and of the muscle anatomy indicates that the remotor muscles are entirely responsible for the rearward axial displacement. The functional origin of the remotors deep within the ventral "sucker" of the adjacent posterior segment provides a firmly fixed anchor point from which are generated the propulsive forces causing water movement. In addition, it is almost certain that the proximal parapodial muscles act to transmit the tensile force of the remotors *directly* to the anterior wall, although the proximal parapodial muscles do not perceptibly shorten during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Post Circ M
AXIAL DISPLACEMENT
Promotor M
Obl Para M
RECOVERY STROKE
POWER STROKE
DISK SHAPE
Prox Para M
Dist Para M
Remotor M
AXIAL DISPLACEMENT
Remotor M
Prox Para M

FIGURE 12. Proposed muscle action during pump cycle, see text for details; periods of muscle shortening indicated by broad lines; maintenance of maximal contraction, narrow lines; active or passive resistance to elongation, dashed lines; passive elongation, omitted.

this phase. It is not possible to state, however, whether the proximal parapodial muscles resist being stretched by actively maintaining tonus, or by simply being elongated to their maximum length. Contraction of the remotor muscles appears to be responsible for inflating the lateral (parapodial) margin and keeping it closely pressed against the inner margin of the tube during the power stroke (*i.e.*, in the extended "disk shape" configuration). [For any muscle set to cause such marginal inflation, its contraction must force coelomic fluid from the main cavity into the peripheral region *without* simultaneously reducing the circumference of the segment. Close inspection of Figure 11 reveals that during the latter phases of the recovery stroke, the posterior wall rapidly loses its hemispherical compound curvature and assumes a more flattened shape. The volume of the coelomic fluid "lost" from the main cavity consequent upon this configuration change is the likely source of fluid required to inflate the parapodial margin. The criss-crossing slips of the remotor muscles are the only fibers whose shortening can accomplish this shape change in the main cavity]. The final shape of the margin, however, cannot be determined solely by the remotor muscles. The integument in this region is likewise too thin and elastic to maintain such a shape by itself. Rather, the numerous distal parapodial fibers, traversing the parapodial cavity axially, must act as "guy wires" to limit the inflation of the marginal flange. Once again, whether they do this actively (by maintaining some contractural tonus) or passively (being simply at the limit of their stretched condition) is not known.

The first event whch occurs at the beginning of the recovery stroke is the axial collapse of the parapodial rim. This action is brought about by the contraction of the distal and proximal parapodial fibers, and results in the expulsion of fluid formerly contained within the margin back into the main coelomic chamber. Starting at the same time, but requiring longer to complete, is a backward folding and radial retraction of the parapodial rim. This is brought about by shortening of the posterior radial and anterior circular fibers. Subsequently, at least five sets of muscles are involved in the basic shape change of the main chamber from disk to sphere configuration. These are the anterior and posterior circular, oblique parapodial, anterior radial, and transverse muscles. The forward axial displacement is brought about by contraction of the promotor muscles, with the probable aid of the oblique parapodial fibers. When forward recovery is complete, the transition from sphere to disk shape is caused by the initial phases of contraction of the remotor muscles.

Discussion

The mechanism by which *Chaetopterus* pumps water through its tube involves the coordinated activities of segments 14, 15, and 16. Each of these pumping segments acts functionally as a "piston" to drive a quantity of water axially within the tube. No differences could be detected in the skeletomusculature of the three piston segments, although the segments themselves are obviously not "identical" since they bear different positional relationships to one another. The majority of muscles run as thin sheets of parallel fibers immediately beneath the fragile, membranous integument. Even those muscles which originate as relatively massive "bundles" (*i.e.*, remotors and promotors), eventually flatten to thin sheets at their insertions. The proximal, oblique, and distal parapodial muscles remain as distinct fibers throughout their courses, and they are the only muscle groups to run from wall to wall across the coelomic cavity. As with any hydrostatic skeletal system, the musculature is responsible for two fundamental actions: (1) small-magnitude displacements, which help to determine segmental shape, and (2) large-magnitude displacements, which cause segmental motion (Chapman, 1950, 1957). The functional organization of the piston segments of *Chaetopterus* may be examined by assigning each muscle group to the following categories, as appropriate: (1) muscles causing axial displacements; (2) muscles causing radial displacements; (3) muscles operative during the power stroke; and (4) muscles operative during the recovery stroke (refer to Fig. 12). Comparison of categories 1 and 2 indicates that many more individual sets of muscles contribute to the radial displacements than contribute to the axial displacements. This, in part, may reflect the fact that radial expansion and retraction is basically a multiple-axis movement, while displacement back and forth along the tube is a uniaxial movement. Comparison of categories 3 and 4 reveals a similar unequal distribution, with far more individual muscle groups actively contracting during the recovery stroke than during the power stroke. However, it is a moot point whether the recovery-phase motions *per se* are more complex than those of the power phase. In this case mere numbers may be misleading since the single muscle set responsible for the power stroke is the largest and most complex of all those involved in pumping water.

The basic motion of the piston segments is reciprocative, and the primary displacements lie along the same axis as the water flow. In addition, there exists a set of secondary (radial) displacements perpendicular to the water flow. By virtue of their precise coordination, these two sets of displacements provide the motions required for an operational positive-displacement pump—namely: (1) an initial centrifugal displacement, which culminates in the formation of a tightly-sealed "piston head" across the tube opening; (2) a power-stroke axial displacement, which moves the piston head and a quantity of water lengthwise down the tube; (3) a centripital displacement, which allows water to by-pass the piston head from the inlet end of the tube; and (4) a recovery-stroke axial displacement, which moves the piston head back to its initial starting position. It should be noted that the separation of piston-forming motions from water-propelling motions is paralleled, in each segment, by the (topographical) separation of the functional "piston ring" (which is limited to the outermost marginal rim) from the functional "piston head" (which encompasses virtually the entire posterior surface). The foregoing analysis applies equally well to the integrated 3-segment pump as to the individual components. The mechanical characteristics which are only applicable to the integrated pump mechanism will be discussed when the kinetics and hydrodynamics of the system are considered (S. C. Brown, in preparation).

The biomechanical characteristics outlined above for *Chaetopterus* stand in sharp contrast to the majority of annelid water-propulsion systems which are based upon peristalsis or undulation. In the latter cases, the basic motion of the functional piston surface approximates a simple transverse wave propagated axially, and the primary displacements (in the radial direction) are perpendicular to the flow of water. These simple radial displacements simultaneously accomplish both piston-forming and water-propelling functions. In addition, any surface which acts as a "piston ring" (or functional seal) also acts at some point in the pump cycle as a "piston head." These characteristics are seen even in those worms whose primary peristaltic irrigation movements are complicated by simultaneous axial displacements (*e.g.*, in *Sabella pavonina;* Mettam, 1969).

Perhaps the most distinguishing feature of members of the family Chaetopteridae is the diversity in their segmental structure. The extreme case is *Chaetopterus,* whose bodily organization strongly parallels the arthropod pattern of grouping adjacent segments into functional units (= "tagmatization"). It seems not unreasonable to suggest that the evolution of the unique pumping mechanism described above was made possible by the possession of a hydrostatic skeleton and, at the very least, facilitated by the tendency towards tagmatization. The exact origin of this pumping mechanism is not clear. Barnes (1964, 1965) appears to be the only investigator to have examined the other genera of chaetopterids with reference to their mechanisms of water-propulsion. Although those genera (*Spiochaetopterus, Telepsavus, Phyllochaetopterus*) considered to be least modified from their presumed spionid ancestors propel water entirely by ciliary means, the more advanced genera (*Ranzanides, Mesochaetopterus*) are reported to irrigate their tubes by a muscular "peristalsis" of many pumping segments "modified somewhat differently" from the three pump segments of *Chaetopterus* (Barnes, 1965, p. 231). In view of the fundamental differences between "peristalsis" and the reciprocating positive-displacement pump mechanism described herein, a closer study of the latter two genera is clearly merited.

I am indebted to Dr. Kenneth P. Able, Dr. Robert D. Allen and Mr. Robert Speck for their knowledgeable advice and for their gracious loans of photographic equipment; and to Mr. Edward Donnelly for his able assistance in maintaining the animals. This investigation was supported in part by New York State Research Foundation grant 20-H101A.

SUMMARY

1. The skeletomusculature of the water-pumping segments of *Chaetopterus variopedatus* was examined in detail and the behavior of the segments during active water-propulsion was analyzed using cinemaphotographic techniques.

2. The musculature of the pumping segments consists largely of thin sheets of radial, transverse, and circular fibers, located immediately beneath the thin integument. Exceptions to this are the numerous isolated fibers running axially across the coelomic cavity of the parapodial rim and the major remotor muscles which originate in the neuropodial "sucker" of the adjacent posterior segment.

3. The motion of the pump segments is basically reciprocative, with the axial displacement during power and recovery strokes approximating a sine function. Centrifugal radial displacements (during the power stroke) effectively seal the lumen of the tube; centripital radial displacements (during the recovery stroke) allow water from the inlet side of the tube to by-pass the pump segment.

4. Comparison of the muscle anatomy with the displacements and shape changes which take place during water-propulsion permitted analysis of the muscle actions during the pump cycle. It is concluded that: (a) both axial and radial displacements during the power stroke are caused by a single set of muscles (remotors); (b) maintenance of the extended-disk configuration is consequent upon coelomic fluid being forced into the parapodial rim, with the axial muscle fibers acting as guy-wires to resist overexpansion; (c) most of the sheet-like muscle groups contract during the recovery stroke, thereby causing the segment to assume a nearly spherical configuration; and (d) two sets of muscles (promotors, oblique parapodial) are responsible for the axial displacement during recovery.

5. The water-pumping mechanism of *Chaetopterus* is compared to those of the majority of worms, which are based on persistaltic or undulatory movements.

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REAPPRAISAL OF PROCTODONE INVOLVEMENT IN THE HORMONAL REGULATION OF LARVAL DIAPAUSE

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Recent findings of juvenile hormone (JH) involvement in larval diapause may form the basis of a revised neuroendocrine theory of larval diapause regulation. It has now been independently confirmed that the mature larval diapause of the southwestern corn borer, *Diatraea grandiosella* Dyar, and the rice stem borer, *Chilo suppressalis* Walker, is initiated and maintained by JH (Chippendale and Yin, 1973; Yin and Chippendale, 1973; Yagi and Fukaya, 1974). These diapause larvae retain actively secreting corpora allata, and the circulating JH enforces inactivity in the brain-ecdysial gland system. Neural and/or neurosecretory axons originating in the brain probably regulate the secretory activity of the corpora allata during diapause. We are now trying to determine exactly how the brain controls the corpus allatum.

As a first step, we have reexamined whether an abdominal hormone, proctodone, controls the activity of the cerebral neurosecretory system of diapause larvae. The ileal epithelium of the european corn borer, *Ostrinia nubilalis* (Hübner), has been named as an endocrine center which responds to photoperiodic signals by the rhythmical secretion of proctodone thereby controlling cerebral neurosecretory activity and diapause development and prepupal morphogenesis (Beck, 1964, 1968; Beck and Alexander, 1964a, b; Beck, Colvin and Swinton, 1965; Beck Shane, and Colvin, 1965; Alexander and Fahrenbach, 1969). The proctodone-cerebral neurosecretory interaction was considered to be the first step of diapause development with proctodone involvement ceasing once the cerebral neurosecretory system was fully activated to secrete ecdysiotropin. Briefly, this hypothesis states that the onset and regulation of diapause is regulated by the phase relationships of an 8 hour proctodone secretory rhythm (phase set by the onset of darkness) and an 8 hour cerebral neurosecretory rhythm (phase set by the onset of illumination). Proctodone is said to activate the neurosecretory system under long days (16L:8D, diapause averting) when the two rhythms are in phase, but not under short days (12L:12D, diapause inducing) when the two rhythms are out of phase. This two oscillator model was used to explain the photoperiodic regulation of the larval diapause of *O. nubilalis* (Beck, 1968).

Although this proctodone function has received wide publicity, the existence of the hormone has never been independently confirmed. McLeod, Graham and Hannay (1969) have cast doubt on its validity and Beck (1974a) did not include it in his new photoperiodic determination model of insect development and diapause. We therefore undertook the present study in an attempt to resolve whether such a hormone originates in the larval ileum of *D. grandiosella* and *O. nubilalis* as a regulator of diapause development and prepupal morphogenesis.

MATERIALS AND METHODS

Test larvae

Nondiapause and diapause larvae (6th instar) of *D. grandiosella* and diapause larvae (5th instar) of *O. nubilalis* were used for the experiments. These crambid and pyrausid moths are both now assigned to the family Pyralidae and enter diapause at the end of their last larval instars. Laboratory-reared *D. grandiosella* were obtained from our stock culture which is maintained on an artificial diet (Chippendale, 1975). Nondiapause larvae were reared at 30° C 12L:12D, reached maturity around 14 days, remained spotted, and pupated shortly thereafter. Diapause larvae were reared at 23° C 12L:12D and reached maturity around 40 days. Prediapause spotted larvae ecdyse into immaculate larvae with pigment-free integument to mark the onset of diapause (Chippendale and Reddy, 1972, 1973). Field-collected diapause southwestern corn borers were also used. These specimens weighed about 300 mg each and were collected from their overwintering site in the root crowns of corn plants from the delta region of southeastern Missouri in December 1973 (mid-diapause larvae) and September 1974 (newly-diapaused larvae). Newly-diapaused larvae of the european corn borer, weighing about 100 mg each, were collected from corn stalks in southeastern Missouri in September 1974, and central Missouri in October 1974. Unlike *D. grandiosella, O. nubilalis* does not have any morphological characteristics to mark the onset of its larval diapause. The non-feeding diapause larvae of both species were held individually in sterilized 23 × 85 mm glass vials containing moist paper strips. The number and origin of the larvae used in the experiments are listed with the results.

Abdominal ligature and nerve cord severance experiments

The effect of a silk thread ligature placed between the 6th and 7th abdominal segments on the rate of diapause termination was examined (Beck and Alexander, 1964b). This ligature removed the ileum, the proposed site of proctodone secretion, from the anterior compartment. Larvae ligatured between the 9th and 10th abdominal segments served as controls. The ventral nerve cord was also severed between the 6th and 7th segments to isolate the terminal abdominal ganglion from the nervous system. The nerve cord was ruptured with a pair of fine forceps and the operation caused little extraneous tissue damage. The following diapause larvae, including untreated controls, ligatured test and control groups, and in two cases nerve cord severed groups, were used: field-collected *D. grandiosella* held at 30° C 24L:OD and 30° C 12L:12D, 60 day old immaculate laboratory-reared *D. grandiosella* held at 30° C 24L:OD, and *O. nubilalis* held at 30° C 15L:9D. An additional untreated control group of *O. nubilalis* was held under a short day, 30° C 12L:12D. These three regimes permitted differing rates of diapause development (Chippendale and Reddy, 1973; Beck and Alexander, 1964b). Larvae were observed daily until all the individuals in the test groups had either died or pupated.

Bioassay of ileal extracts

A bioassay of ileal extracts of *D. grandiosella* was conducted to determine whether they accelerated the pupation rate of diapause larvae (Beck *et al.,* 1965b).

The ileal epithelium was dissected out of mature 14–16 day old nondiapause larvae, stripped of surrounding muscles, rinsed free of hemolymph, and stored in a sterile 0.85% saline solution at − 20° C. Samples were collected shortly after the onset of the photophase because the proctodone hypothesis predicts that the epithelial cells should then be packed with hormone-containing granules when larvae are held under a 12L : 12D photoperiod (Beck *et al.*, 1965a). After being homogenized, the ileal preparation was centrifuged for 20 min at 15,000 g at 0–5° C. The concentration of the resulting supernatant was then adjusted to 0.5, 1.0 or 1.5 ileal equivalents/5 μl saline and immediately injected into the abdomen of field-collected diapause larvae. Larvae which served as solvent controls received 5 μl of the sterile saline solution. The bioassay was conducted as follows: prior to receiving the injection the test larvae were preconditioned at 30° C 12L : 12D until about 5% had pupated. They were then injected, transferred to 25° C 12L : 12D to permit a lower rate of diapause development, and observed for 30 days. If the ileal extract bypassed the environmental regulatory system the test larvae should have pupated more rapidly than those in the control.

Histology of the ileum

The histology of 16 day old nondiapause mature larval and newly-ecdysed pupal ileum was compared using standard procedures. Anterior intestines were dissected out of larvae at the beginning of the photophase and out of white pupae which had molted about 2 hours earlier. They were fixed in aqueous Bouin's solution, embedded in paraplast, and cut into a series of 6 μ thick transverse sections. The sections were stained with a paraldehyde fuchsin solution (Ewen, 1962), and photographed using a Wild M20 system and Panatomic X film.

Fluorescence microscopy of ileal epithelium

The proctodone hypothesis suggests that the hormone is released into the hemolymph at the beginning of the scotophase and approximately 8 hr thereafter in an ultradian rhythmical fashion. This rhythm was established by examining the autofluorescence of fresh ileal epithelium. The autofluorescence was correlated with the presence of paraldehyde fuchsin-positive granules and proctodone activity in the ileal cells (Beck *et al.*, 1965a). We examined the ileal epithelium of mature larvae of both *D. grandiosella* and *O. nubilalis* for both autofluorescence and acridine orange (AO)-induced fluorescence. For autofluorescence observations, ileal epithelium was removed, washed, cut longitudinally, flattened out on microscope slides, and immersed in 0.85% saline solution. The epithelium was examined under a Wild M20 fluorescence microscope and photographed using Tri X film. A similar procedure was carried out for AO fluorescence observations, with the exception that the newly dissected ileal epithelia were immersed in an AO in saline solution (0.005%) for 30 min. Observations were made according to the proposed empty and full cell stages of the proctodone secretory rhythm for larvae held at 30° C 12L : 12D. Preparations were set up about 2 hr from the end of the photophase when most of the ileal epithelia should have exhibited autofluorescence, at the beginning of the scotophase, and 4 hr into the photophase when the autofluorescence in many of the preparations should have been at least partially quenched.

Electron microscopy of ileal epithelium

Ileal cells of mature 16 day old nondiapause larvae which displayed bright autofluorescence were fixed in 3% glutaraldehyde in Sorenson's buffer (pH 7.2) for 3 to 4 hours. They were post-fixed in 2% buffered osmium tetroxide for 3 to 4 hr at 4° C and then dehydrated and embedded in Spurr's epoxy resin. After only 30 min of glutaraldehyde fixation and prior to post-fixation, some cells were incubated in a medium to detect acid phosphatase activity using β-glycerophosphate as the substrate (Brunk and Ericsson, 1972). Controls were run by adding sodium fluoride (10 mM) as an inhibitor to the incubation medium. Sections ($<$ 1000 Å) were cut with a Reichert ultramicrotome, placed on 200 mesh copper grids, and stained with a 2% alcoholic uranyl acetate for 25 min and, for the non-incubated tissues, alkaline lead citrate for 5 min at room temperature (Venable and Coggeshall, 1965). Electron micrographs were taken using an RCA-EMU-3G instrument operated at 100 KV.

RESULTS

Larval response to abdominal ligatures and nerve severance

Figure 1 illustrates the response of diapause larvae of *D. grandiosella* and *O. nubilalis* to a ligature applied between the 6th and 7th abdominal segments. If, as is suggested by the proctodone hypothesis, the ileum is the hormonal source for terminating diapause, the test larvae should remain in diapause. Figure 1A shows that untreated field-collected larvae of *D. grandiosella* held at 30° C 24L : OD reached 50% pupation in 22 days whereas larvae in the ligatured test and control groups did not even attain this mark. Only 32% of the former and 20% of the latter pupated, and the remaining larvae died during the 38 days of observation. Similar results were obtained when field larvae were exposed to 30° C 12L : 12D (Fig. 1B). In this case the untreated controls reached 50% pupation in 39 days, and only 12% of the ligatured test larvae and 8% of the ligatured control larvae pupated. The remaining larvae died within the 68 days' observation period. Severing the ventral nerve cord between the 6th and 7th abdominal segments resulted in a higher pupation rate than in the ligatured test and control groups, but still caused a high mortality rate. The results show that 68 days after treatment 38% of the larvae had pupated, leaving the remainder dead. Figure 1C illustrates the effects of the abdominal ligature on laboratory-reared *D. grandiosella* exposed to a 30° C 24L : OD regime. As before, only 16% of the ligatured test larvae and 2.5% of the ligatured control larvae survived to pupate, whereas 50% of the untreated controls had pupated in 35 days. We must therefore conclude that these results do not confirm the existence of any factor in the 7–9th abdominal segments whose exclusion from the anterior compartment would prevent pupation. The high mortality rate in the ligatured groups was probably due to severe stress caused by the partitioning of the hemocoel. However, more ligatured test than ligatured control larvae pupated in all 3 experimental situations.

Figure 1D summarizes the results of the abdominal ligature and nerve cord severance experiment conducted on diapause larvae of *O. nubilalis* held at 15L : 9D. The results show that 50% of the untreated larvae pupated in 50 days. Larvae held in another untreated control group at 30° C 12L : 12D (n = 40, not

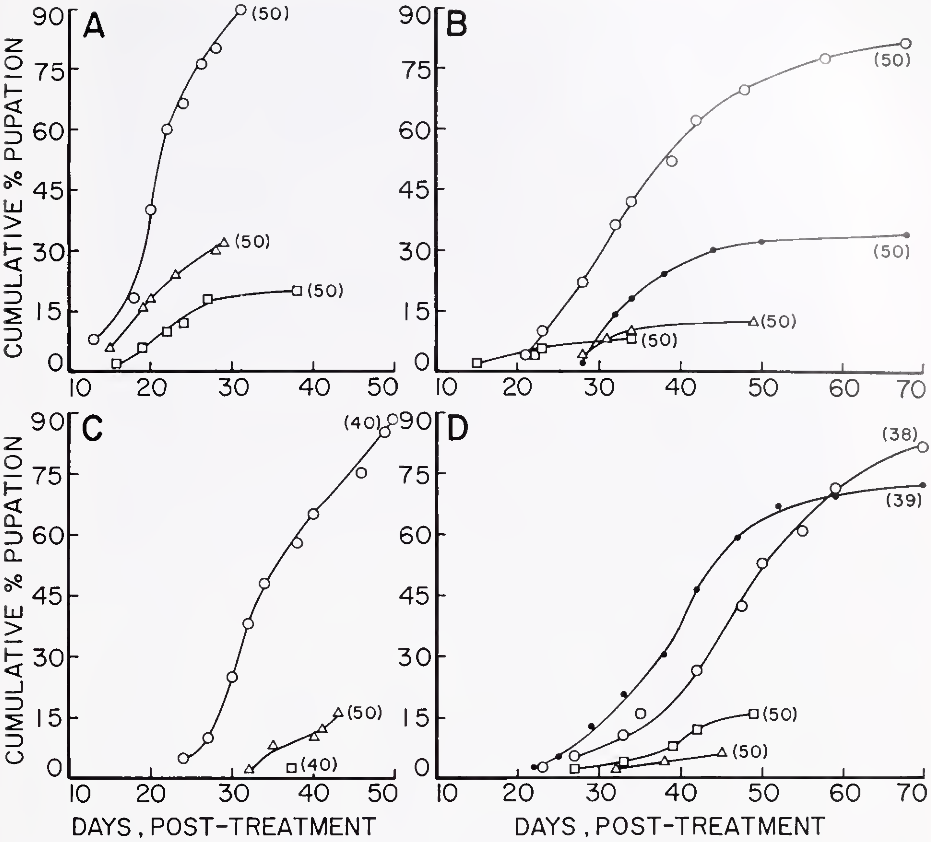


Figure 1. Effect of abdominal ligatures and nerve cord severance on the rate of diapause development of *Diatraea grandiosella* and *Ostrinia nubilalis*. A) Field-collected *D. grandiosella* larvae (Dec. 1973) maintained in a 30° C 24L:OD regime; B) field-collected *D. grandiosella* larvae (Dec. 1973) maintained in a 30° C 12L:12D regime; C) laboratory-reared *D. grandiosella* larvae (60 days old) maintained in a 30° C 24L:OD regime; and D) field-collected *O. nubilalis* larvae (Oct. 1974) maintained in a 30° C 15L:9D regime. Open circles represent untreated control; open triangles represent larvae ligatured between 6th and 7th abdominal segment (test); open squares represent larvae ligatured between 9th and 10th abdominal segment (control); closed circles represent larvae whose nerve cord was severed between 6th and 7th abdominal segment. Parenthetic numbers refer to the initial number of larvae in each group.

illustrated) did not pupate during 70 days of observation, thereby confirming the pronounced effect of photoperiod on diapause development of these Missouri-collected borers (McLeod and Beck, 1963). In contrast, many of the ligatured test and control larvae died within 70 days, and only 6% of the former and 15% of the latter pupated. These data again show that the ligature is often lethal to the larvae. No significant differences were observed between the ligatured test and control groups. We therefore did not obtain any evidence that larval diapause of *O. nubilalis* is prolonged by a ligature applied between the 6th and 7th abdominal

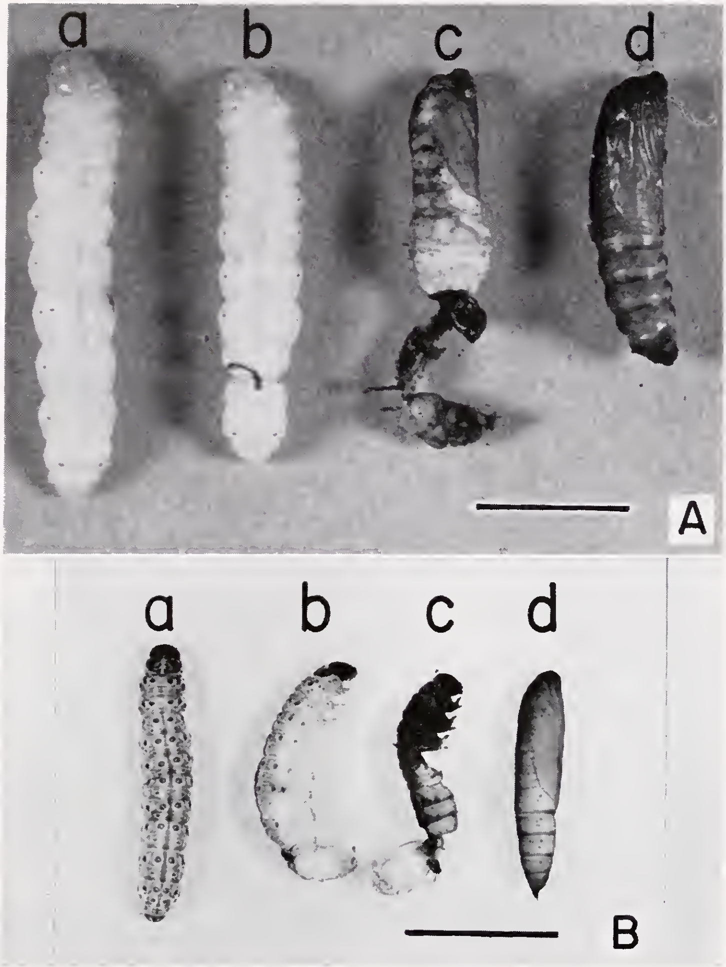


FIGURE 2. Larvae and pupae of the southwestern (A) and european (B) corn borers illustrating the abdominal ligature experiment. (a) Shows normal diapause larva; (b) shows diapause larva ligatured between the 6th and 7th abdominal segments; (c) shows pupa formed following the abdominal ligature; and (d) shows normal pupa. Scale bars equal 1 cm.

segments. Larvae whose nerve cord was severed survived well and pupated at a slightly higher rate than the untreated controls. This treatment resulted in a lower mortality in the european than in the southwestern corn borer (*cf.* Fig. 1B). While the significance of this result is not yet clear, it is possible that isolating the terminal abdominal ganglion from the central nervous system frees the brain from an inhibitory neural influence which normally is involved in sustaining diapause.

Figure 2 illustrates the four classes of *D. grandiosella* and *O. nubilalis* observed in the ligature experiment and also serves to compare the larval and pupal forms of the two species. Mature southwestern corn borers are about 3 times as large

TABLE I

Effect of an ileal extract from mature nondiapause larvae on diapause development of the southwestern corn borer.

Larvae* (number)	Regime	Treatment‡	30 days post-treatment		
			Larvae (%)	Pupae (%)	Mortality (%)
65	30° C 12L:12D	Untreated control	55	22	23
65	25° C 12L:12D	Untreated control	91	5	4
25	25° C 12L:12D	Solvent control	80	4	16
35	25° C 12L:12D	0.5 Ileal equiv./L	84	11	5
40	25° C 12L:12D	1.0 Ileal equiv./L	85	5	10
36	25° C 12L:12D	1.5 Ileal equiv./L	83	3	14

* Diapause larvae collected from the field in September 1974 were used. They were preconditioned for 38 days at 30° C 12L:12D until about 5% had pupated, before the remaining larvae were divided into the experimental groups.

‡ Each test larva received an injection of 5 μl of an ileal saline extract prepared from mature nondiapause larvae (14–16 days old).

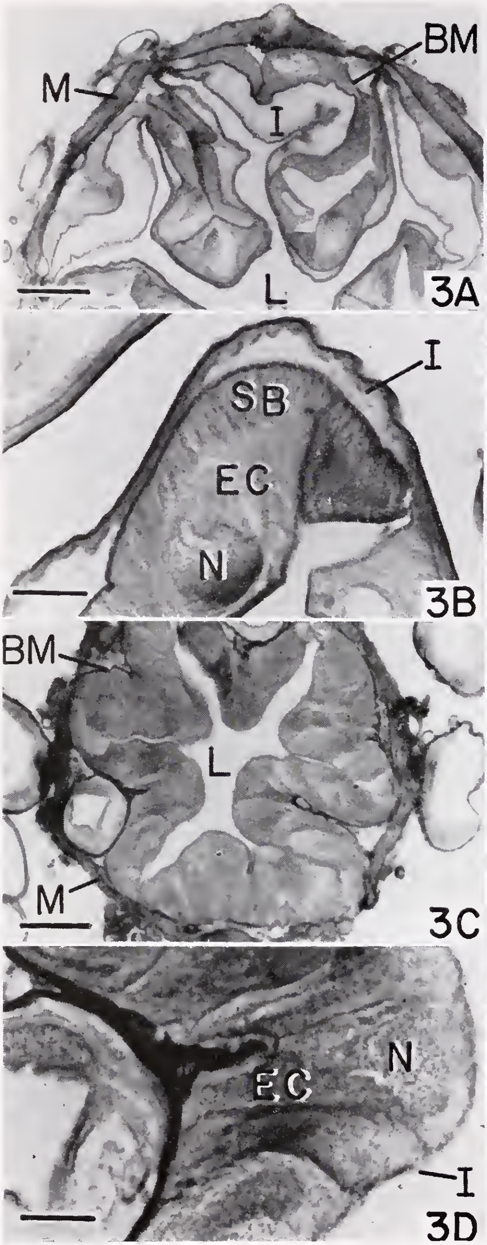
as the equivalent european corn borers. The immaculate diapause southwestern corn borer is illustrated. The spotted nondiapause and prediapause morphs differ only in the presence of pigmented integumental pinacula.

Response of diapause larvae to an ileal extract

A proctodone bioassay which had previously given positive results for *O. nubilalis* was performed on *D. grandiosella* (Beck *et al.,* 1965b). This bioassay was crucial in the formulation of the original proctodone hypothesis since it provided the only direct evidence for the existence of an abdominal hormone. Table I shows that the injection of ileal extracts did not accelerate diapause development. Since 22% of the larvae in the untreated control held at 30° C 12L:12D pupated compared with only 5% of those transferred to 25° C 12L:12D, the 25° C regime was stringent enough to uncover any proctodone effect. The results show, however, that 30 days after treatment no significant differences were found between the number of larvae and pupae in the 2 control and 3 test groups. The few pupal molts recorded at 25° C occurred periodically over the 30 day observation period. The injection of an ileal extract did not bypass the normal environmental programming of diapause development, and therefore no proctodone effect was detected.

Histology of the larval and pupal ileum

The larval proctodeum of both species is divided into the pyloric valve and ileum (anterior intestine) and the rectum. The histology of the ileum of *O. nubilalis* larvae has already been described (Beck *et al.,* 1965b). We examined the comparative histology of the mature larval and newly-ecdysed pupal ileum of *D. grandiosella* (Fig. 3). This borer's mature larval ileum is similar to that of *O. nubilalis* and is made up of large squamous epithelial cells which are present



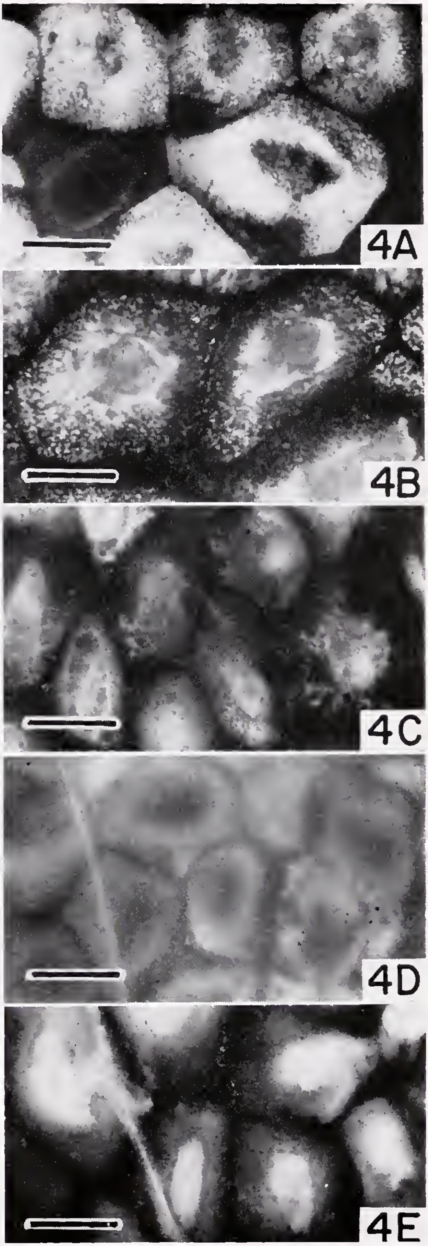


FIGURE 3. Histological sections of the ileum from mature larvae (A, B) and newly molted pupae (C, D) of the southwestern corn borer. Symbols used are: BM, basement membrane; EC, epithelial cell; I, intima; L, lumen; M, circular muscle; N, nucleus; SB, striated border. Scale bars equal 40 μ (A and C), 10 μ (B and D).

FIGURE 4. Autofluorescence and acridine orange-induced fluorescence in the ileal epithelium of the southwestern and european corn borer. A, B, show ileum from late stage mature

in 6 longitudinal infoldings in the empty gut (Fig. 3A). A cuticular intima, distinct nuclei and striated borders in the epithelial cells, basement membrane, and circular musculature are clearly visible (Fig. 3B). Although these sections were prepared from an ileum which exhibited autofluorescence, no distinct paraldehyde-fuchsin positive granules were detected in the cytoplasm. The presence of autofluorescence and paraldehyde-fuchsin positive granules has been correlated with the retention of proctodone activity in the ileum of *O. nubilalis* (Hassemer and Beck, 1969).

In contrast to the larval ileum, the pupal ileum of *D. grandiosella* is much smaller and has a different histological structure (Fig. 3C, D). The infoldings of the epithelial cells, though still present, were less pronounced. An indistinct intima was present, and the columnar epithelial cells lacked a striated border. Deposits of paraldehyde-fuchsin positive granules were not detected. These findings show that the ileum undergoes substantial changes at the beginning of metamorphosis. It is likely that the proposed secretory function of the mature larval ileum is associated with these cellular changes.

Fluorescence of ileal epithelium

Since an ultradian cycle of autofluorescence in the ileum is believed to correlate with the secretion of proctodone (Beck *et al.*, 1965a), we examined the ileal epithelium of *D. grandiosella* and *O. nubilalis* for the presence of both auto and induced fluorescence (Fig. 4). Figure 4A illustrates autofluorescent particles in the ileal epithelium of nondiapause mature larvae of *D. grandiosella.* Ileal epithelium displaying bright green autofluorescence was much more common in pharate pupae than in mature larvae. Figure 4B shows AO-induced fluorescence in the ileal epithelium of a mature nondiapause larva of *D. grandiosella.* This preparation also displayed autofluorescence before AO treatment and the location of the cytoplasmic AO-positive particles corresponded to the autofluorescent ones (*cf.* Fig. 4A). Following this AO treatment a green fluorescence was induced in the nucleus and an orange one in the cytoplasm indicating that the fluorescent cytoplasmic particles are probably lysosomes (Allison and Young, 1969). Figure 4C illustrates AO-induced fluorescence in the ileal epithelium of an early diapause larva of *O. nubilalis.* The nucleus fluoresced green and the cytoplasmic the orange induced fluorescent particles. We conclude that they are lysosomes Figure 4D shows the absence of autofluorescent material in the ileum of *D. grandiosella* while Figure 4E shows that only fluorescent nuclei are detected in the non-autofluorescent epithelium which had been incubated with AO. These last observations confirm that the green autofluorescent particles correspond with the orange induced fluorescent particles. We conclude that they are lysosomes which are presumably involved in remodeling the ileum at the onset of metamorphosis.

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nondiapause southwestern corn borers displaying autofluorescence (A) and acridine orange-induced fluorescence (B); C, ileum from field-collected diapause european corn borer (Sept. 1974) displaying acridine orange-induced fluorescence; D, E, ileum from mature nondiapause southwestern corn borers showing absence of autofluorescence (D), and acridine orange-induced fluorescence in cell which lacked autofluorescence (E). Scale bars equal 40 μ.

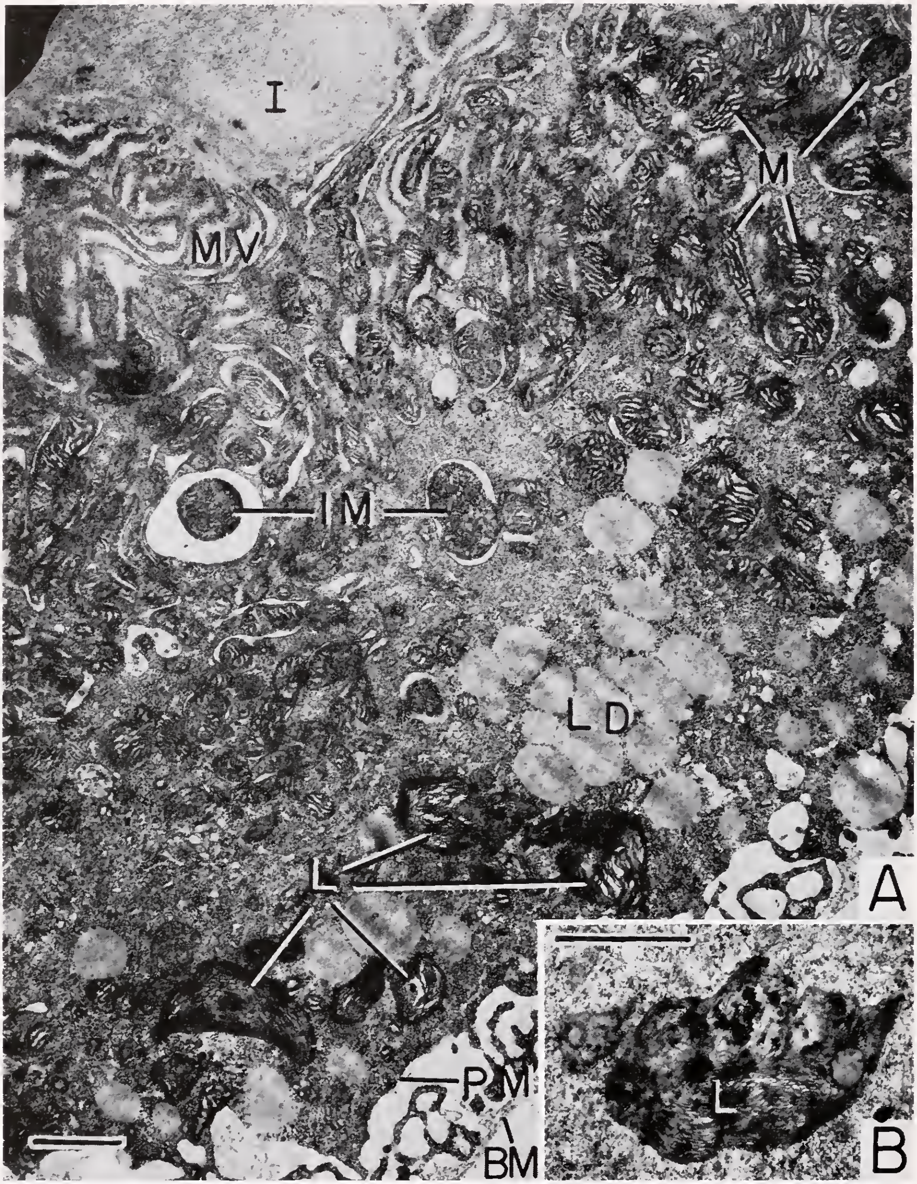

FIGURE 5. Fine structure of an autofluorescent ileal cell of a mature nondiapause southwestern corn borer (A), and an ileal lysosome following a histochemical test for acid phosphatase activity (B). Symbols used are: BM, basement membrane; I, intima; IM, isolated mitochondrion; L, lysosome; LD, lipid droplet; M, mitochondrion; MV, microvilli; PM, plasma membrane. Scale bars equal 1 μ.

Ileal tissue was prepared for fluorescence microscopy at periods when the cells should have been both empty and full of autofluorescent particles (Beck *et al.*, 1965a). Although in both species we observed cells which displayed cytoplasmic fluorescence and others which were completely devoid of fluorescence, we were unable to detect any pattern of fluorescence which corresponded to an eight hour autofluorescence rhythm. However, a rigorous study of the rhythmical appearance and disappearance of the autofluorescence was not undertaken and these observations are based upon ileal preparations from about 50 southwestern and 25 european corn borers. Consequently, while our findings suggest that an underlying rhythmicity regulates the appearance of these fluorescent particles, we did not obtain any convincing evidence to support the existence of an eight hour ultradian rhythm which is phase-set by the onset of darkness.

Ultrastructure of ileal epithelium

The electron microscope was used to examine the cytoplasmic particles of the ileal epithelium of mature southwestern corn borers which had ceased feeding. Figure 5A shows a typical preparation from an autofluorescent ileum of a mature nondiapause larva. From the lumen side the cell contains: cuticular intima; mitochondria, associated with the microvilli of the striated border or within isolation bodies; lipid droplets; lysosomes; infolded plasma membrane; and is bounded by a basement membrane. The identity of the laminated membranous organelles as lysosomes was confirmed following the acid phosphatase procedure (Fig. 5B). No lead deposits were seen in the organelles of cells which had been incubated with the acid phosphatase inhibitor, sodium fluoride. The organelles contained lead precipitates deposited at local sites of acid phosphatase activity and therefore have a hydrolytic function which is characteristic of lysosomes. This finding provides additional evidence to show that lysosomes are present in the ileum prior to the onset of metamorphosis.

Discussion

Our results do not support the hypothesis that the ileal cells of the southwestern and european corn borers produce a hormone which interacts with the cerebral neuroscretory system during diapause or at the onset of metamorphosis. Although detailed results were not presented, a proctodone effect in the regulation of the larval diapause of the pine moth, *Dendrolimus pini,* and the codling moth, *Laspeyresia pomonella,* was also ruled out (Kind, 1968; Peterson and Hamner, 1968).

The proposed rhythmical interaction between proctodone and the cerebral neurosecretory system served as the basis for an ultradian two oscillator model of diapause and development (Beck, 1964). During the past 10 years, however, additional research has led to a revision of this model. A most convincing argument for reappraisal came from an experiment in which > 50% of european corn borer larvae reared under a noncircadian photoperiod of 16L:16D entered diapause. Since under this regime the postulated proctodone and cerebral neurosecretory rhythms should have been in phase, growth and development of the borer should have proceeded without the intervention of diapause (Beck, 1974a). The two oscillator model has now been superseded by a developmental determination model which better accounts for our existing knowledge about the photoperiodic control of insect development and diapause (Beck, 1974a, b).

Important questions remain concerning the post-digestive functions of the ileum, and whether any abdominal system exerts a regulatory influence on diapause and development. Our finding that the mature larval ileum contains lysosomes confirms the observations of Hassemer and Beck (1968, 1969) and McLeod *et al.* (1969). We also show that the fluorescent particles are lysosomes, thereby providing an alternative explanation for the "proctodone" phenomenon. Lysosomes are known to autofluoresce (Allison and Young 1969), and frequently to be associated with paraldehyde-fuchsin positive material (De Duve and Wattiaux, 1966). The lysosomes observed in the mature larval ileum are presumably autolysosomes which may discharge enzymes and lytic products into the hemolymph (De Duve and Wattiaux, 1966; Lockshin, 1969). This exocytosis would account for the observed secretory activity of the ileum. Furthermore, histological observations usually show that the lepidopteran pupal ileum contains less cellular material than the larval one (Judy and Gilbert, 1970). The mature larval ileum also appears to function as an osmoregulatory center (Hassemer and Beck, 1971). We observed mitochondria associated with the microvilli of the ileum of *D. grandiosella* larvae, and this system presumably actively transports water and ions even during diapause. Osmoregulation encompassing both transport and secretion may represent the principal function of the ileum in post-feeding larvae.

A neural or neurosecretory involvement of the insect abdomen in endocrine regulation of growth and metamorphosis has not yet been detailed. It is possible that the abdominal ganglia provide some essential input to the larval brain to signal the beginning of metamorphosis. Evidence is beginning to suggest that neural signals originating in the abdomen prime the brain to initiate metamorphosis (Edwards, 1966, 1967; Sehnal and Edwards, 1969; Beck, 1970). We have undertaken a preliminary study of the possible involvement of the abdominal ganglia in regulating the onset of pupation of the southwestern corn borer. Although we have not yet obtained any reproducible effects, we recognize that the timing of any possible neural signals is critical and plan to investigate the system further.

We believe that receptors in the larval head receive the photoperiodic and thermal signals which regulate the onset and termination of a facultative diapause (Geispits, 1957; Williams and Adkisson, 1964; Claret, 1966). This exteroceptive input to the brain may be supplemented and modified by proprioceptive input from the abdomen providing information about size, form, and posture (Edwards, 1967; Nijhout and Williams, 1974). The brain then integrates these afferent signals to institute a diapause or nondiapause program. In the case of a facultative mature larval diapause, the larval brain may continue to activate the corpora allata leading to a continued secretion of JH and the institution and maintenance of diapause (Yin and Chippendale, 1973). Larval diapause is probably terminated when environmental signals received by the brain lead to a change in the efferent signal to the corpora allata, resulting in a decline in the hemolymph JH titer, and the initiation of the larval-pupal molting cycle.

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SUMMARY

1. No evidence was found that the larval ileum of the corn borers, *Diatraea grandiosella* and *Ostrinia nubilalis,* secretes a developmental hormone, "proctodone."

2. An abdominal ligature which isolated the ileum of diapause larvae of both species caused high mortality but did not retard the pupation rate.

3. Extracts prepared from the ileal epithelium of mature nondiapause larvae of *D. grandiosella* were injected into diapause larvae and did not cause premature diapause termination.

4. Marked cytological changes occurred in the ileum at the onset of metamorphosis. At times the ileal epithelium of both species displayed auto and acridine orange-induced fluorescence, characteristic of lysosomes.

5. An electron microscopic examination of the autofluorescent ileal epithelium of *D. grandiosella* revealed organelles which had typical lysosomal features and stained positively for acid phosphatase activity.

6. The secretory activity of the ileum can be accounted for by lysosomal involvement in its metamorphic reorganization, and by its osmoregulatory functions.

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COLCHICINE, CYTOCHALASIN B, AND PIGMENT MOVEMENTS IN OVARIAN AND INTEGUMENTARY ERYTHROPHORES OF THE PRAWN, *PALAEMONETES VULGARIS*[1]

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The erythrophores of the prawn, *Palaemonetes vulgaris,* are controlled by pigment-concentrating and pigment-dispersing hormones (Brown, Webb, and Sandeen, 1952). These hormones appear to exert their primary actions at the plasma membrane, not entering the cells to initiate pigment migration (Fingerman and Connell, 1968; Fingerman, 1969).

In several vertebrates and the fiddler crab, *Uca pugilator,* drug studies have implicated both microtubules (Wright, 1955; Malawista, 1965, 1971a; Wikswo and Novales, 1969; Schliwa and Bereiter-Hahn, 1973; Lambert and Crowe, 1973) and microfilaments (Malawista, 1971b; McGuire and Moellmann, 1972; Lyerla and Novales, 1972; Lambert and Crowe, 1973; Robison and Charlton, 1973) as cell structures possibly involved in pigment granule movement within chromatophores. Colchicine and cytochalasin B have been favorite tools for investigators studying microtubules and microfilaments because colchicine can dissociate microtubules (Borisy and Taylor, 1967) and cytochalasin B has as one effect the disruption of microfilaments (Schroeder, 1970; Wessells, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn, and Yamada, 1971).

Practically all the investigators who have studied the effect of colchicine on chromatophoric pigment migration have observed that this drug inhibits pigment aggregation (*i.e.,* concentration). The notable exception as far as the experiments to be described below are concerned is the report by Robison and Charlton (1973) who stated that they observed no inhibition of pigment aggregation in the dark erythrophores on the surface of the ovary of the prawn, *Palaemonetes vulgaris,* after these cells were treated with colchicine. However, at the time Robison and Charlton published their data, we had already performed some unpublished experiments with integumentary erythrophores of this prawn and found that colchicine did indeed inhibit pigment concentration in these chromatophores. A series of experiments was then devised to reconcile the difference between the observations of Robison and Charlton and our unpublished observations. In addition, the effects of cytochalasin B and colchicine on pigment dispersion in this prawn were determined. Robison and Charlton reported that cytochalasin B inhibited pigment aggregation in *Palaemonetes,* but neither they nor anyone else has previously tested the effect of either colchicine or cytochalasin B on pigment dispersion in the erythrophores of this prawn. Aside from the report of Robison and Charlton, there is no other publication dealing with the effects of these drugs on chromatophores of *Palaemonetes vulgaris.*

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MATERIALS AND METHODS

The specimens of the prawn, *Palaemonetes vulgaris,* used in the experiments described below, were collected in the vicinity of Woods Hole, Massachusetts, by members of the Supply Department of the Marine Biological Laboratory. The Hogben and Slome (1931) scheme was used to stage the erythrophores. According to the Hogben and Slome system, stage 1 represents maximal pigment concentration, stage 5 maximal dispersion, and stages 2, 3, and 4 the intermediate conditions. Prawns with maximally concentrated red pigment were obtained by placing intact individuals in white containers for at least one hour, whereas prawns with maximally dispersed red pigment were obtained by putting individuals into black containers for a similar period of time.

The erythrophores used in these experiments were observed *in vitro.* In some experiments the erythrophores on the surface of the ovaries were used, in others we used the erythrophores of the epidermis attached to the portion of the exoskeleton dorsal to the heart. The ovarian erythrophores were obtained by carefully removing the ovaries from the prawns. To obtain the integumentary chromatophores, a portion of the carapace approximately 3 mm long and 4 mm wide was removed and then cut into two pieces, each about 3 × 2 mm, one serving as the experimental piece, the other as the control. These portions of isolated carapace consisted of exoskeleton and chromatophore-containing epidermis. The pieces of carapace and ovaries were placed in Pantin's crustacean saline (Pantin, 1934) that was diluted until it was isosmotic with the blood of the prawn (Fingerman and Connell, 1968).

Colchicine (Sigma) was prepared in isosmotic saline. Cytochalasin B (Aldrich) was first dissolved in dimethyl sulfoxide (DMSO) and this solution was then diluted with isosmotic saline to a cytochalasin B concentration of 10 μg/ml in a 0.1% DMSO solution.

Red pigment-concentrating hormone was obtained by preparing extracts of the tritocerebral commissure in a concentration of 0.1 tritocerebral commissure equivalent per 0.025 ml. This commissure contains red pigment-concentrating hormone, but no red pigment-dispersing hormone (Brown, Webb, and Sandeen, 1952). Red pigment-dispersing hormone without red pigment-concentrating hormone was obtained by subjecting abdominal nerve cords to gel chromatography in essentially the manner described by Fingerman and Bartell (1973). The abdominal nerve cord has the highest ratio of red pigment-dispersing hormone to the antagonistic concentrating hormone (Brown, Webb, and Sandeen, 1952). By means of gel chromatography these hormones can be separated from each other (Fingerman and Bartell, 1973). Twenty-five abdominal nerve cords were extracted in 0.3 ml distilled water, applied to an 0.8 × 31.0 cm Bio-Gel P-6 column, and eluted with distilled water. One ml fractions were collected and made isosmotic with the prawn's blood by the addition of 0.18 ml of 400% Pantin's saline. The fractions were tested for red pigment-dispersing activity and the three having maximal activity were pooled for use in these experiments. Appropriate assays also revealed that these three fractions lacked pigment-concentrating activity.

The ovaries and pieces of integument were placed in the depressions on white porcelain spot plates. Each depression contained 0.05 ml of saline or other appropriate solution, depending upon the experiment, and one ovary or one

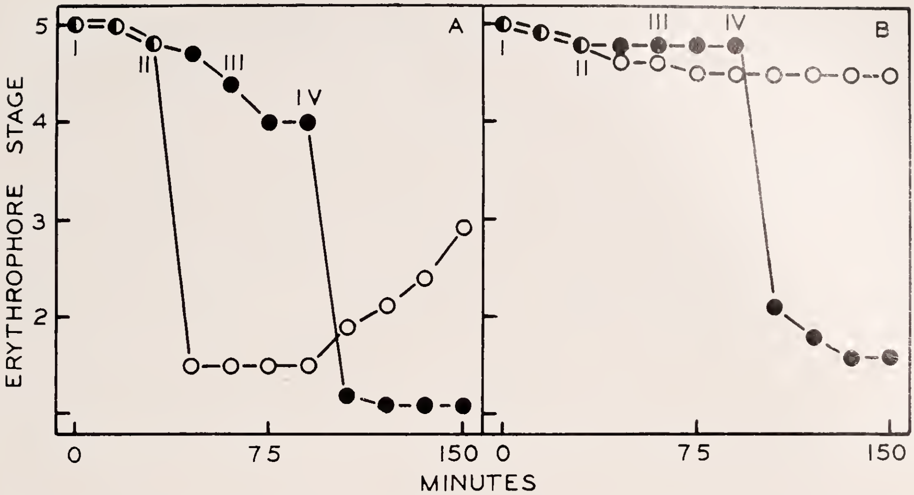

FIGURE 1. Relationships between integumentary erythrophore stage and time in minutes. A: circles represent erythrophores in saline at outset; dots, erythrophores in colchicine (25 mM) at outset. The pigment in these erythrophores was initially maximally dispersed. At the outset (at I) the erythrophores were placed in either colchicine or saline. Red pigment-concentrating hormone (RPCH) was administered to both the saline-exposed and colchicine-treated erythrophores after 30 minutes (at II). The colchicine and RPCH were replaced by saline 60 minutes after 0 time (at III) and RPCH was administered again 90 minutes after 0 time (at IV) to the erythrophores that had been in the colchicine. B: all of the erythrophores (circles and dots) were exposed to colchicine (25 mM) at the outset (at I). All received RPCH after 30 minutes (at II). Half of the erythrophore-containing pieces of carapace (dots) were placed in saline 60 minutes after 0 time (at III), and 90 minutes after 0 time (at IV) RPCH was again administered to the erythrophores that had been but were no longer in colchicine.

piece of integument. When, according to the protocol, a hormone or other solution was added to a depression containing an ovary or piece of integument and 0.05 ml of saline or other solution, the additional volume was always 0.025 ml. In every experiment either five experimental and five control ovaries or five experimental and five control pieces of integument were used. Each experiment was performed twice. Consequently, each data point in the following figures represents the mean of 10 determinations.

EXPERIMENTS AND RESULTS

Effect of 25 mM *colchicine on integumentary erythrophores*

The object of the first series of experiments was to determine the effects of 25 mM colchicine on pigment concentration and pigment dispersion in the integumentary erythrophores. The first experiment of this series was designed to determine, first of all, whether 25 mM colchicine inhibits pigment concentration in the integumentary erythrophores of *Palaemonetes vulgaris.* At the outset (at I in Fig. 1A) one set of pieces of exoskeleton with the adhering erythrophores was placed in 25 mM colchicine for 30 minutes and a second set into saline. Then (at II in Fig. 1A), 0.025 ml of the tritocerebral commissure extract containing the red pigment-concentrating hormone was added to all the pieces. It is clear from Figure 1A that there was much inhibition of the pigment-concentrating response (II–III in Fig. 1A) on the part of the erythrophores exposed to the 25 mM colchicine compared with those that had been in saline alone until the hormone was added. Fifteen minutes after the hormone had been added, the pigment in the erythrophores that had been in the saline was nearly maximally concentrated whereas the erythrophores in the 25 mM colchicine were virtually unchanged after 15 minutes exposure to the hormone. Then, as part of the same experiment, we wished to see whether the effect of the colchicine was reversible. To accomplish this aim, 60 minutes from the start of the experiment (at III in Fig. 1A) the erythrophores in the colchicine were transferred to saline alone in order to wash out the drug. The saline was changed every five minutes for 30 minutes and then (at IV in Fig. 1A) the washed pieces of exoskeleton with the adhering erythrophores were placed in 0.05 ml saline to which was added 0.025 ml of the tritocerebral commissure extract and the erythrophores were observed for one more hour. The erythrophores that had been exposed to the colchicine and then washed were able to respond nearly maximally to the red pigment-concentrating hormone, thus revealing that the effect of the colchicine was reversible. The pigment in the control erythrophores (they had not received the 90-minute dose of hormone given to the washed erythrophores) began to redisperse toward the end of this experiment. Presumably, most or all of the hormone added originally had become inactivated and the red pigment began to disperse spontaneously in the absence or near absence of pigment-concentrating hormone.

An experiment was then designed to demonstrate in another fashion that 25 mM colchicine inhibits concentration of the pigment in these integumentary erythrophores. From the outset (at I in Fig. 1B) all of the pieces of exoskeleton with the adhering erythrophores were exposed to 0.05 ml of 25 mM colchicine for 30 minutes (I–II in Fig. 1B) and then 0.025 of the tritocerebral commissure extract was added to each piece (at II in Fig. 1B). It is obvious from Figure 1B (between II and III), just as from Figure 1A, that the colchicine inhibited the response to red pigment-concentrating hormone. After the erythrophores had been exposed to the hormone for 30 minutes, one-half of the pieces of exoskeleton with the adhering erythrophores was placed in saline (at III in Fig. 1B). The saline was changed every five minutes for 30 minutes in order to wash out the colchicine. After this half hour of washing, the washed pieces were placed in 0.05 ml saline to which was added 0.025 ml of the tritocerebral commissure extract (at IV in Fig. 1B). The unwashed pieces were simply placed into another 0.05 ml of 25 mM colchicine to which was added 0.025 ml of the tritocerebral commissure extract. The washed erythrophores then responded to the hormone. Again the effect of the colchicine was reversible.

The next experiment was similar to that of Figure 1A except that there were two changes in the procedure; namely, we used erythrophores that initially had maximally concentrated instead of dispersed pigment and red pigment-dispersing hormone instead of the pigment-concentrating hormone. These changes were introduced in order to determine, first of all, whether 25 mM colchicine inhibits pig-

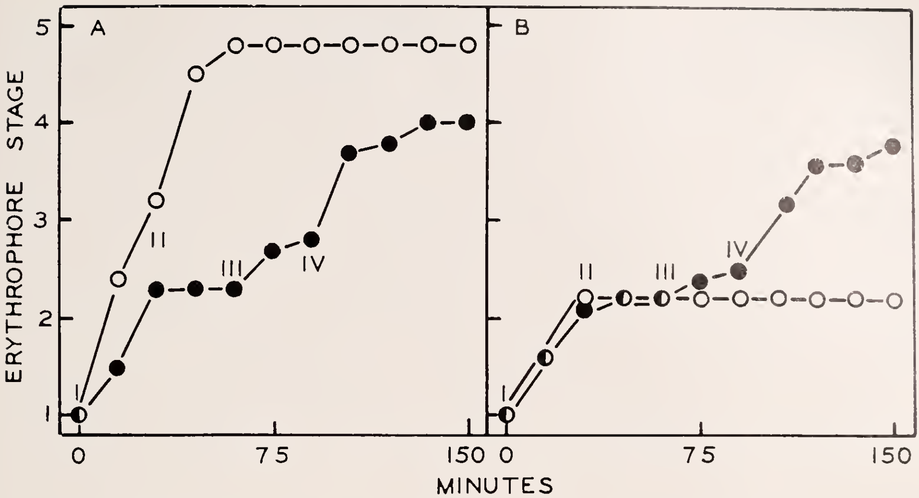

FIGURE 2. Relationships between integumentary erythrophore stage and time in minutes. A: circles represent erythrophores in saline at outset; dots, erythrophores in colchicine (25 mM) at outset. The pigment in these erythrophores was initially maximally concentrated. At the outset (at I) the erythrophores were placed in either colchicine or saline. Red pigment-dispersing hormone (RDPH) was administered to both the saline-exposed and colchicine-treated erythrophores after 30 minutes (at II). After 60 minutes from 0 time (at III) the erythrophores that had been in colchicine were washed in saline. RPDH was readministered 90 minutes after 0 time (at IV) to the erythrophores that had been in colchicine. B: all of the erythrophores (circles and dots) were exposed to colchicine (25 mM) at the outset (at I). All received RPDH after 30 minutes (at II). Half of the erythrophore-containing pieces of carapace (dots) were placed in saline 60 minutes after 0 time (at III), and 90 minutes after 0 time (at IV) RPDH was again administered to the erythrophores that had been but were no longer in colchicine.

ment dispersion in these integumentary erythrophores. One set of pieces of exoskeleton with the adhering erythrophores was placed in 25 mM colchicine and a second set in saline (at I in Fig. 2A). After 30 minutes, red pigment-dispersing hormone was administered to all of the pieces (at II in Fig. 2A) and the erythrophores were observed for 30 minutes. Then the pieces in colchicine were washed in saline (III–IV in Fig. 2A) as described above for 30 minutes and then given the hormone again (at IV in Fig. 2A). It is apparent from inspection of Figure 2A that the colchicine inhibited pigment dispersion in response to the hormone and this effect was reversible just as in the case of pigment concentration. Furthermore, the inhibitory effect of colchicine on pigment dispersion was apparent even before the hormone was administered. The spontaneous pigment dispersion that occurs when erythrophores with maximally concentrated pigment are removed from the body (I–II in Fig. 2A) was less in the erythrophores in the colchicine than in those in the saline. The dispersion that was observed in the colchicine-treated erythrophores probably occurred before sufficient colchicine had entered the cells to inhibit the pigment-dispersing mechanism.

The final experiment (Fig. 2B) of this series was the counterpart of that described for Figure 1B. In this experiment all the pieces of exoskeleton were

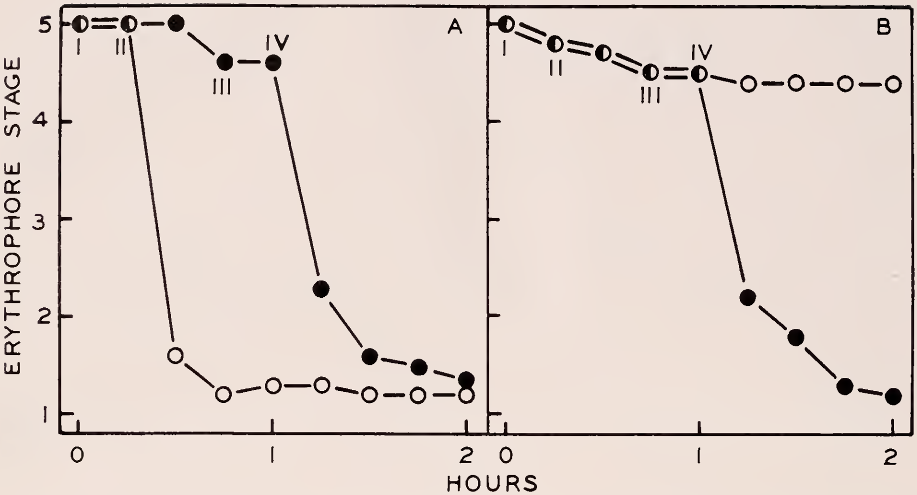

FIGURE 3. Relationships between integumentary erythrophore stage and time in hours: A: circles represent erythrophores in 0.1% dimethyl sulfoxide (DMSO) at outset; dots, erythrophores in cytochalasin B (10 μg/ml) in 0.1% DMSO at outset. The pigment in these erythrophores was initially maximally dispersed. At the outset (at I) the erythrophores were placed in either cytochalasin B or DMSO. Red pigment-concentrating hormone (RPCH) was administered to both the DMSO-exposed and cytochalasin B-treated erythrophores after 15 minutes (at II). The cytochalasin B was replaced by 0.1% DMSO 45 minutes after 0 time (at III) and RPCH was administered again 60 minutes after 0 time (at IV) to the erythrophores that had been in the cytochalasin B. B: all the erythrophores (circles and dots) were exposed to cytochalasin B (10 μg/ml) at the outset (at I). All received RPCH after 15 minutes (at II). Half of the erythrophore-containing pieces of carapace (dots) were placed in 0.1% DMSO 45 minutes after 0 time (at III), and 60 minutes after 0 time (at IV) RPCH was again administered to the erythrophores that had been but were no longer in cytochalasin B.

placed in the 25 mM colchicine solution at the outset (at I in Fig. 2B) and given the red pigment-dispersing hormone after 30 minutes (at II in Fig. 2B). Thirty minutes later half of the pieces were washed for 30 minutes in saline (III–IV in Fig. 2B) and given the hormone again (at IV in Fig. 2B). The unwashed pieces were simply placed into fresh colchicine solution to which was added the hormone (at IV in Fig. 2B). This experiment, as in Figure 2A, showed that colchicine inhibited red pigment dispersion in response to the hormone and that this inhibition was reversible.

Effect of cytochalasin B on integumentary erythrophores

The object of this series of experiments was to determine whether cytochalasin B can inhibit pigment migration in the prawn's integumentary erythrophores and if so, whether the effect is reversible. In the first experiment the effect of this drug on pigment concentration was determined. Pieces of exoskeleton with the adhering erythrophores were taken from prawns that had been kept in black containers. One set of pieces was placed into 0.05 ml of the cytochalasin B solution and the remainder served as a control, each control piece being put into 0.05 ml of 0.1%

DMSO (at I in Fig. 3A). After 15 minutes (at II in Fig. 3A) 0.025 ml of tritocerebral commissure extract was added. The tritocerebral commissure extracts used in these experiments with cytochalasin B were prepared in the same concentration ($\frac{1}{10}$ equivalent per dose) as in the colchicine experiments but in 0.1% DMSO in saline instead of in saline alone. It is clear from Figure 3A that the cytochalasin B strongly inhibited the response to the red pigment-concentrating hormone (II–III in Fig. 3A). Then, 45 minutes after the start of the experiment the pieces that had been in the cytochalasin B were placed into 0.1% DMSO and every five minutes for 15 minutes the DMSO solution was changed (III–IV in Fig. 3A). Finally, one hour after the experiment had started, the washed erythrophores were exposed to tritocerebral commissure extract again (at IV in Fig. 3A). The washed erythrophores then responded to the red pigment-concentrating hormone. The inhibition produced by cytochalasin B is clearly reversible, just as that produced by colchicine.

The next experiment (Fig. 3B) is comparable to the colchicine experiment of Figure 1B. Integumentary erythrophores from prawns in black containers were exposed to cytochalasin B for 15 minutes (I–II in Fig. 3B), pigment-concentrating hormone was added (at II in Fig. 3B), and the expected inhibition was observed (II–III in Fig. 3B). Then, 45 minutes after the experiment started (at IV in Fig. 3B), half the pieces of exoskeleton were washed for 15 minutes in 0.1% DMSO and then given the hormone again (at IV in Fig. 3B). The unwashed pieces were simply given fresh cytochalasin B plus the hormone (at IV in Fig. 3B). The washed erythrophores responded to the pigment-concentrating hormone, showing again the reversibility of the inhibition.

The final two experiments of this series (Fig. 4) utilized erythrophores that initially had maximally concentrated red pigment. In Figure 4A are shown the data obtained when one-half the pieces of exoskeleton were placed in 0.05 ml cytochalasin B and the remainder in 0.1% DMSO (at I in Fig. 4A) for 15 minutes at which time (at II in Fig. 4A) 0.025 ml of the red pigment-dispersing hormone solution was administered to each of the pieces. The response of the erythrophores in the cytochalasin B was strongly inhibited (II–III in Fig. 4A). The erythrophores in the cytochalasin B were then washed between 45 and 60 minutes from the start of the experiment in 0.1% DMSO (III–IV in Fig. 4A) and then the hormone was readministered to the washed erythrophores (at IV in Fig. 4A). The washed erythrophores then responded to the hormone. This experiment showed that the pigment-dispersing response of these erythrophores can be inhibited by cytochalasin B and that the effect is reversible. Furthermore, the inhibitory effect of the cytochalasin B on pigment dispersion was apparent even before the hormone was administered (I–II in Fig. 4A) as was also the case with the colchicine in Figure 2A.

In the next experiment all of the pieces of exoskelton with the adhering erythrophores were placed in cytochalasin B for 15 minutes (I–II in Fig. 4B) and then (at II in Fig. 4B) 0.025 ml of the red pigment-dispersing hormone were added. The pigment-dispersing response had been inhibited once again (II–III in Fig. 4B). Then, between 45 and 60 minutes from the start of the experiment (III–IV in Fig. 4B) half of the pieces were washed in 0.1% DMSO and then given another dose of the hormone (at IV in Fig. 4B). The unwashed

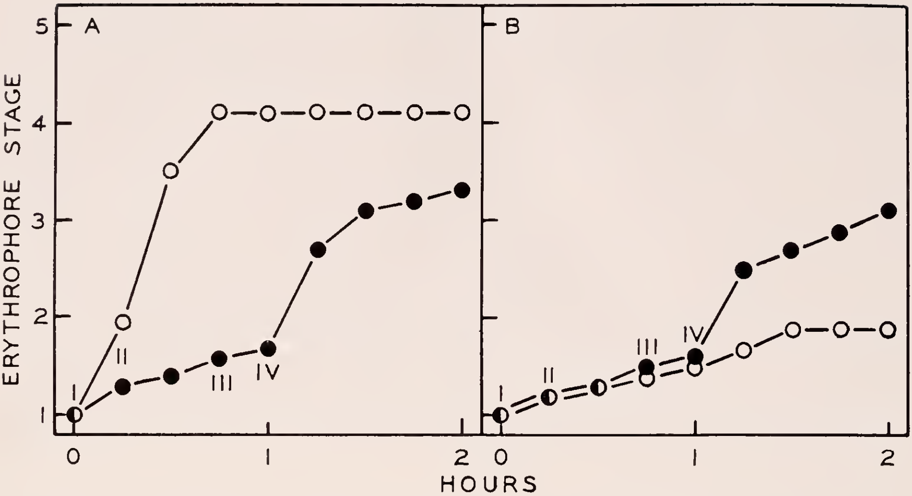

FIGURE 4. Relationships between integumentary erythrophore stage and time in hours. A: circles represent erythrophores in 0.1% dimethyl sulfoxide (DMSO) at outset; dots, erythrophores in cytochalasin B (10 μg/ml) at outset. The pigment in these erythrophores was initially maximally concentrated. At the outset (at I) the erythrophores were placed in either cytochalasin B or DMSO. Red pigment-dispersing hormone (RPDH) was administered to both the DMSO-exposed and cytochalasin B-treated erythrophores after 15 minutes (at II). The cytochalasin B was replaced by 0.1% DMSO 45 minutes after 0 time (at III) and RPDH was readministered 60 minutes after 0 time (at IV) to the erythrophores that had been in the cytochalasin B. B: all of the erythrophores (circles and dots) were exposed to cytochalasin B (10 μg/ml) at the outset (at I). All received RPDH after 15 minutes (at II). Half of the erythrophore-containing pieces of carapace (dots) were placed in 0.1% DMSO 45 minutes after 0 time (at III), and 60 minutes after 0 time (at IV) RPDH was again administered to the erythrophores that had been but were no longer in cytochalasin B.

erythrophores were simply placed in fresh cytochalasin B (at IV in Fig. 4B) to which was added 0.025 ml of the hormone solution. Only the washed erythrophores were capable at that time of dispersing their pigment.

Effect of 5 mM *colchicine on integumentary erythrophores*

The data presented in Figure 1 clearly reveal that 25 mM colchicine inhibits the response of integumentary erythrophores of the prawn, *Palaemonetes vulgaris*, to red pigment-concentrating hormone. However, Robison and Charlton (1973), as stated above, reported that colchicine does not inhibit the pigment-concentrating response in ovarian erythrophores of this prawn. Aside from the different source of the erythrophores in the prawn, integument versus ovary, there was one other very important difference between our experiments and those of Robison and Charlton. Whereas we had used a 25 mM solution of colchicine, Robison and Charlton had used only a 1 mM solution. It seemed possible that rather than there being a fundamental difference between these erythrophores from different parts of the prawn, the reason for the difference in results was due simply to the much lower concentration of colchicine that Robison and Charlton used. The following experiment was performed to examine this possibility.

Pieces of exoskeleton with the adhering erythrophores from prawns from black pans were placed into a 5 mM solution of colchicine (0.05 ml) while the control pieces were simply placed into saline. Thirty minutes later 0.025 ml of the tritocerebral commissure extract were added to each depression. The 5 mM colchicine did not inhibit the pigment-concentrating response of these erythrophores. Similarly, 5 mM colchicine did not inhibit the response of erythrophores of prawns taken from white containers to red pigment-dispersing hormone administered after the erythrophores had been incubated for 30 minutes in the drug.

Effect of colchicine on ovarian erythrophores

The final experiment was performed to confirm the supposition that the difference between our results and those of Robison and Charlton (1973) was due to the difference between the concentrations of colchicine solutions that were used and not to a fundamental difference between the pigment-concentrating mechanisms of the integumentary and ovarian erythrophores of this prawn. Four groups of ovaries were removed from prawns that had been kept in black containers so that their ovarian erythrophores would contain maximally or nearly maximally dispersed pigment. Each ovary of one group was placed into 0.05 ml 25 mM colchicine, each of the second was put into 0.05 ml of 1 mM colchicine, and each ovary of the remaining two groups was placed in 0.05 ml saline alone. Thirty minutes later, 0.025 ml of the tritocerebral commissure extract containing red pigment-concentrating hormone was added to each ovary in the colchicine solutions and to each of those in one of the two groups in saline. To each ovary in the remaining group in saline were simply added an additional 0.025 ml of saline. The erythrophores were then observed for one hour. The averaged results are presented in Figure 5 where it can be seen that the red pigment in the erythrophores in saline alone remained virtually maximally dispersed, that the pigment in the erythrophores in saline that received the tritocerebral commissure extract concentrated strongly, that the erythrophores in 25 mM colchicine showed virtually no response to the hormone, and that the erythrophores in the 1 mM colchicine responded to the red pigment-concentrating hormone but not quite as strongly as did the erythrophores in saline that received the hormone.

DISCUSSION

It is clear from Figure 5 that the response of ovarian erythrophores to red pigment-concentrating hormone is greatly inhibited by 25 mM colchicine. The obvious explanation for the discrepancy between these data and the results of Robison and Charlton (1973) who reported that the pigment-concentrating response of ovarian erythrophores in *Palaemonetes vulgaris* is not inhibited by colchicine is that these investigators used a solution (1 mM) that was not concentrated enough to inhibit the erythrophores. Our results with integumentary erythrophores are, however, in agreement with their observation that cytochalasin B inhibits pigment concentration in ovarian erythrophores.

Both the pigment-dispersing and pigment-concentrating responses of the integumentary erythrophores of this prawn are inhibited by either colchicine or cytochalasin B. *Palaemonetes vulgaris* is the first animal for which it has been

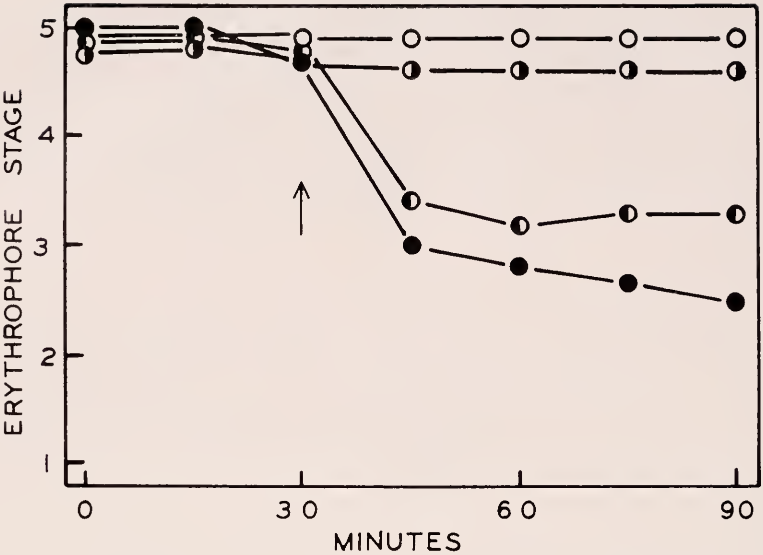

FIGURE 5. Relationships between the stages of erythrophores on the surface of the ovary and time in minutes. Circles represent erythrophores in saline alone; dots, erythrophores in saline from the outset and 30 minutes after 0 time tritocerebral commissure extract containing red pigment-concentrating hormone was added; circles half-filled on left, erythrophores in 1 mM colchicine from outset and 30 minutes after 0 time tritocerebral commissure extract was added; circles half-filled on right, erythrophores in 25 mM colchicine from outset and 30 minutes after 0 time tritocerebral commissure extract was added. The arrow shows when the hormone was added.

demonstrated that both colchicine and cytochalasin B inhibit the two processes of pigment dispersion and aggregation in any of the animal's chromatophores. As stated above, colchicine causes the disruption of microtubules; cytochalasin B, microfilaments. In amphibians colchicine inhibits melanin granule aggregation but not dispersion (Wright, 1955; Malawista, 1965, 1971a), whereas cytochalasin B strongly inhibits melanin dispersion but also has a less pronounced inhibitory effect on melanin granule aggregation (Malawista, 1971b; Magun, 1973). In the fiddler crab, *Uca pugilator,* colchicine inhibits only pigment aggregation while cytochalasin B clearly inhibits both aggregation and dispersion (Lambert and Crowe, 1973). In teleosts microtubules are required for both aggregation and dispersion of the pigment in melanophores (Murphy and Tilney, 1974). Interestingly, in contrast to the effects of cytochalasin B on other chromatophores that have been studied, the drug accelerated both dispersion and aggregation of pigment in the melanophores of the teleost *Oryzias latipes* (Ohta, 1974). In those species where colchicine and/or cytochalasin B inhibit pigment migration it has been previously postulated that microtubules and/or microfilaments respectively provide the propulsive force for movement of the pigment granules. Ohta, on the other hand, in light of his observation that cytochalasin B accelerates pigment migration in *Oryzias latipes,* suggested that in this fish microfilaments normally inhibit melanin granule movement.

Although the effects of colchicine and cytochalasin B on pigment migration have been attributed by previous investigators to disruption by these drugs of microtubules and microfilaments respectively, it is possible that these drugs could have other actions that might result in altering the rate of pigment granule migration. In the protozoan, *Tetrahymena pyriformis,* for example, colchicine impairs the mobility of membrane-intercalating particles, and this mobility does not appear to involve microtubules (Wunderlich, Müller, and Speth, 1973). Furthermore, both cytochalasin B and colchicine inhibited the (Na^+-K^+)-ATPase of rat liver plasma membranes (Bos and Emmelot, 1974). It is quite possible that the inhibition of pigment movement by 25 mM colchicine is not due to microtubule disruption because Robison and Charlton (1973) found that 1 mM colchicine disrupted erythrophore microtubules but had no appreciable effect on pigment aggregation. Recently, we have found (Lambert and Fingerman, manuscript in preparation) that 25 mM lumicolchicine, a derivative of colchicine which does not disrupt microtubules, is as effective as colchicine in inhibiting pigment aggregation in melanophores of the fiddler crab, *Uca pugilator,* which is further indication that the inhibitory action of the high concentrations of colchicine used herein may be at one or more sites other than microtubules. It is unlikely that colchicine is simply exerting a nonspecific poisoning action on these erythrophores because the effect of this drug is rapidly reversible. At this time, it cannot be definitely stated that cytochalasin B is active on microfilaments in erythrophores of *Palaemonetes vulgaris,* the plasma membrane being another possible site of action.

Fingerman (1969) presented a model for the actions of the red pigment-concentrating and pigment-dispersing hormone in *Palaemonetes* at the cellular level. These hormones were visualized as primarily altering the permeability of the cell membrane to the passage of ions, resulting in changes in the concentrations of sodium, potassium, and calcium in the erythrophore. The result of these ion concentration changes is activation of the mechanism that produces migration of the pigment in the appropriate direction that the hormone calls for, either centrifugal or centripetal. Furthermore, cyclic AMP is involved as the second messenger in the pigment-dispersing process (Fingerman, Hammond, and True, 1968). The present results enable us to extend this model one step further. The red-pigment-concentrating hormone through its effect on the potassium-sodium ratio in the cell may activate microfilaments which in turn would provide a propulsive force to the pigment-dispersing hormone through its effect on calcium permeability and/or its stimulation of the synthesis of its intermediary, cyclic AMP, would activate either the same or a different set of microfilaments to produce centrifugal migration of the pigment granules. A similar situation seems to hold for pigment dispersion in amphibian melanophores in which pigment dispersion induced by cyclic AMP is inhibited by cytochalasin B (Novales and Novales, 1972; McGuire, Moellmann, and McKeon, 1972; Magun, 1973; Fisher and Lyerla, 1974). How the propulsive force is actually applied to the granules has not been determined for any animal as yet.

We wish to thank the members of the Supply Department of the Marine Biological Laboratory for providing the prawns.

SUMMARY

1. The effects of colchicine and cytochalasin B on pigment migration in integumentary erythrophores of the prawn, *Palaemonetes vulgaris,* were determined.

2. Colchicine in a concentration of 25 mM inhibited responses to both the red pigment-concentrating and red pigment-dispersing hormones by the integumentary erythrophores. Colchicine at a concentration of 5 mM did not inhibit the responses of these chromatophores to these hormones.

3. Cytochalasin B (10 μg/ml) also inhibited both centrifugal and centripetal migrations of the pigment granules in these integumentary erythrophores.

4. Colchicine in a 25 mM solution almost completely inhibited the response to red pigment-concentrating hormone in ovarian erythrophores whereas 1 mM colchicine produced only a slight inhibition of this response.

5. The integumentary erythrophores of *Palaemonetes vulgaris* are the first chromatophores that have been observed in any animal where both pigment dispersion and aggregation are inhibited by either colchicine or cytochalasin B.

6. These results are discussed in relation to previously published data from this and other laboratories.

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PIGMENTATION IN THE ORANGE TUNICATE, *ECTEINASCIDIA TURBINATA*[1]

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Ecteinascidia turbinata, a colonial ascidian of shallow waters, is characterized by the bright orange pigment in the adults' mantle tissue. The pigment is localized in fusiform organelles, approximately 3 to 6 μ in length, that are organized into stellate shaped masses heavily concentrated at the siphon end and tapering in density toward the base of the zooid, which is almost colorless. Mature oocytes and early embryos of the species are dark yellow to orange, but their pigment is diffuse, not granular, and is solubilized in yolk platelets.

The chemical nature of the pigment in *E. turbinata* has not been determined with certainty. The adult pigment was considered to be carotenoid on the basis of its similarity to the orange pigment in Synoicidae and Botryllidae species (George, 1939). The orange pigment in *Ascidia mentula* (Webb, 1939) and *Phallusia mammilliata* (Endean, 1960), however, has chemical properties entirely different from carotenoids, casting some doubt on the original interpretation based solely on comparative coloration between different species. The pigment in eggs and embryos has likewise not been subjected to direct analysis but designated "lipochrome" on the basis of its solubility in alcoholic solvents (Simkins, 1924).

Also, there has not been agreement as to whether the stellate shaped masses of the mantle pigment are true cells. Nuclei could not be found in these aggregates in hematoxylin stained paraffin sections of mantle tissue, and it was concluded that they were not cells (Simkins, 1924). George (1938), however, found methyl green stained nuclei in orange blood cells of this species and postulated that these lodged in the mantle and became permanent fixtures of this tissue.

The following studies attempt to resolve some of these questions concerning the chemical and cellular nature of the pigment in *E. turbinata.* It was felt that a better understanding of these properties of the pigment might allow some insights as to its functional role and perhaps provide a basis for any subsequent work on this little known pigmentary system.

Materials and Methods

Colonies of zooids were collected from pilings at Longbird Bridge on St. George's Island, Bermuda. They were kept in constantly flowing sea water aquaria at environmental temperatures at the Bermuda Biological Station and used for these studies within one week of their capture.

For small scale, qualitative chemical determinations, individual zooids were stripped of their tests and cut transversely into siphon and basal pieces. The basal ends contained stomach, hepatopancreas, heart, ovotestis, and any developing embryos within the brood pouch, while siphon ends were comprised primarily of mantle and pharyngeal tissue, with dense concentrations of mantle pigment. From 3 to 5 siphon ends were ground in a glass homogenizer with 1 ml of various solvents (see results) and centrifuged at approximately 1000 × *g* for 10 minutes. Yellow supernatant, indicating soluble pigment, was filtered under suction using 0.45 μ pore size Millipore filters. Pigment solutions were chromatographed on 2 × 8 cm cellulose thin-layer sheets (Eastman Chemicals) in various developing solvents. Pigment from whole eggs and embryos was extracted, centrifuged and filtered as described, and stored at −4° C in the dark. Chromatographs of these extracts were made on 2 × 8 cm silica-gel sheets (Eastman Chemicals) predried at 100° C for 30 minutes. The absorption spectra for egg and embryo pigment were determined with a Beckman DU spectrophotometer.

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For large scale batch preparations of mantle pigment, the individual zooids were removed from the colonies and cut into siphon and basal halves. From 400 to 500 siphon halves were constantly stirred for 1 hour at room temperature in 500 ml distilled water. This separated the unpigmented tests from heavily pigmented mantle tissue, which was then rinsed of adhering water with acetone, weighed, and homogenized in 15 to 20 ml of 10% trichloracetic acid in 40% acetone using a motor driven teflon pestle with a glass homogenizer. Homogenates were centrifuged at 1000 × *g* for 15 minutes, and the yellow supernatant filtered with the 0.45 μ Millipore apparatus.

The pigment from these extracts was purified further by first shaking 20 ml of extract over 100 ml chloroform for 24 hours at room temperature, allowing the system to stand for 12 hours at 9° C, and withdrawing the chloroform hypophase which contained no visible pigment. Any remaining acetone in the aqueous epiphase was removed by bubbling with N_2 for 10 minutes. The pigment solution was then washed 4 times with ethyl ether to remove the trichloroacetic acid, and N_2 again bubbled through it until the ether had evaporated. The resulting aqueous extract (about 12 ml) containing some precipitate and at approximately pH 6.0 was dialyzed against 1000 ml of distilled water for at least 24 hours at room temperature and with a minimum of 2 changes of distilled water. This resulted in the accumulation of a flocculent brown precipitate and the loss of yellow color in the dialysis bag. The precipitate was collected by centrifugation, dried over P_2O_5 and redissolved in 50% methanol. The yellow solution was filtered and its absorption spectrum determined on a Cary 15 recording spectrophotometer.

For ultrastructural studies, individual zooids were dissected mechanically and siphon pieces cut into quarters measuring approximately 1 × 3 cm. These were placed immediately into 50 ml of 6% glutaraldehyde and 3% sucrose in 0.1 M phosphate buffer at pH 7.4. They were fixed at laboratory temperatures for 4 to 6 hours, rinsed in 10 ml of cold phosphate buffer for 24 to 48 hours, and postfixed in OsO_4. Uranyl acetate and lead monoxide were used as counter-stains and the sections examined on a JEM-T6S electron microscope.

RESULTS

Chemical analyses

Mantle pigment was not soluble in hydrophobic solvents, such as petroleum ether or hexane, or acetone, but was soluble in organic-aqueous systems includ-

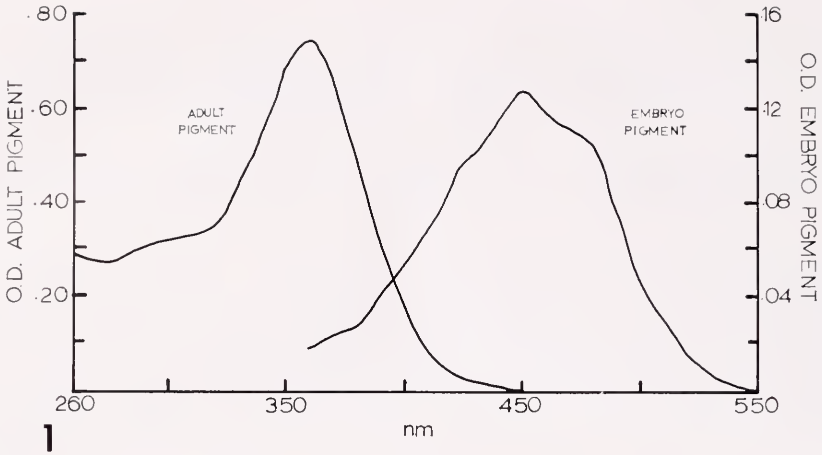

FIGURE 1. Absorption spectra of purified mantle pigment in 50% methanol (left) and embryo pigment (right) in petroleum ether. O. D. scales have been adjusted to provide curves of similar heights.

ing 70% dioxane, 50% methanol acidified to pH 3 with 1N HCl, a mixture of 4 parts butanol : 1 part acetic acid : 1 part water and 10% trichloroacetic acid in 40% acetone (TCA-acetone). Extracts in these solvents provided clear yellow solutions after filtration that remained stable at room temperatures under normal lighting conditions. The TCA-acetone extracted pigment purified as described above and redissolved in 50% methanol migrated as a single fraction of R_f 0.52 on cellulose thin-layer chromatograms using TCA-acetone as the developing solvent. The chromatograms showed no fluorescence, but the yellow pigment absorbed either long or short wave UV irradiation.

The addition of potassium borohydride to the TCA-acetone extracts to a concentration of 1 M decolorized the solution, whereas the addition of 1 M sodium nitrite did not. The TCA-acetone extracted pigment could be dialyzed using TCA-acetone as the dialyzing medium but was retained as a flocculent brown precipitate when dialyzed against distilled water.

The absorption spectrum of dialyzed pigment dissolved in 50% methanol is shown in Figure 1. The pigment absorbs strongly in the UV region, exhibiting a sharp peak at 360 nm. Absorbence in the visible range is general, shows no peaks, and falls to zero at wavelengths greater than 450 nm.

Egg and embryo pigment was soluble in acetone, petroleum ether or hexane. The extracted pigment separated into three visible fractions on silica-gel TLC using a 1 hexane : 3 ethyl acetate mixture as developing solvent. The absorption spectrum in petroleum ether for embryo pigment is shown in Figure 1. There is a major peak of 450 nm, with minor shoulders at 430 and 480 nm. The solubility of egg and embryo pigment in organic solvents, its fractionation on silica-gel chromatograms and absorbence are characteristic of carotenoids (Fox, 1953; Needham, 1974). When petroleum ether extracts of the embryo pigment were shaken with 90% methanol, the pigment remained in the ether epiphase, indicating the

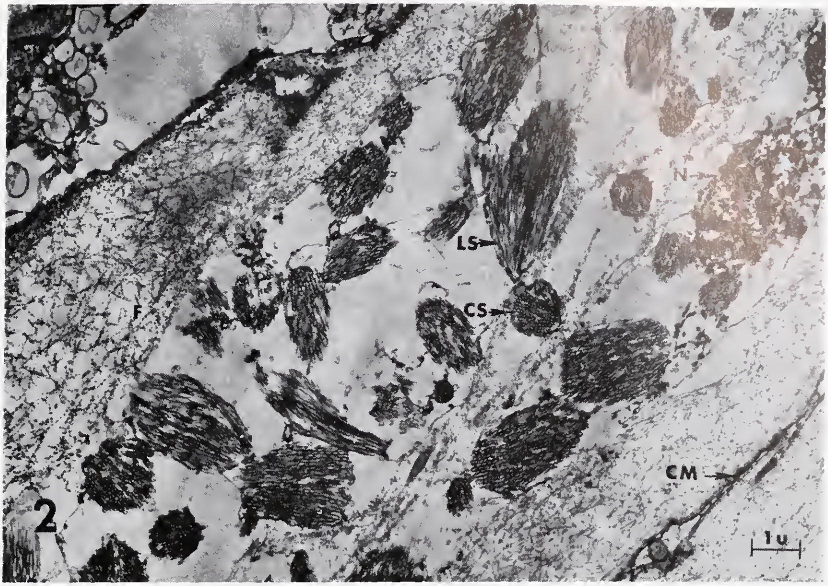

FIGURE 2. Section of pigment body mass in mantle tissue at siphon end. Pigment bodies are seen in association with an apparent nucleus (N) and may be bound by a cytoplasmic membrane (CM). The region surrounding the pigment bodies is composed of fibrils (F). Cross sections (CS) and longitudinal sections (LS) of pigment bodies are found within the same pigment body mass; 8,400 × magnification.

presence of carotenes or xanthophyll esters and the absence of xanthophylls (Fox and Vevers, 1960).

Morphological analyses

Ultrastructural characteristics of pigment bearing elements at the siphon end of the zooid are shown in Figure 2. The fusiform bodies seen at the light microscopic level appear as a structured and orderly array of tubes, approximately 500 to 600 Å in diameter, running parallel to the longitudinal axis of the organelle. It could not be determined from these studies if the tubular elements were continuous and unbranched, but cross sections of tubes from a single body were uniform in diameter (Fig. 2, CS), and this seems a likely possibility. Individual pigment organelles apparently are not bound by a limiting membrane, but this is a questionable point as no attempts were made to vary fixation and embedding procedures, and thus there is no way of determining whether those employed in this study were optimal for the demonstration of perhaps fragile membrane material.

The pigment bodies are discrete elements found within areas that may be cells or remnants of cells. The region surrounding clumps of pigment bodies is composed of a meshwork of fibrils bound by an apparent cytoplasmic membrane. The bodies are usually located in fibril free channels and occasionally associated with an apparent nucleus (Fig. 2, N).

Discussion

The orange pigment in *E. turbinata* is intense and a striking characteristic of this species. Both embryos and adults have similar coloration but differ in the morphological appearance of the pigment—diffuse in the embryo and granular in adults. The granular pigment is also found within the tissues of the ovotestis, where maturing oocytes contain their complement of diffuse pigment, giving this organ a bright orange color.

The presence of granular pigment in follicle cells surrounding development oocytes and the diffuse pigment in the larger prospective ova led Simkins (1924) to postulate that eggs became pigmented by a transfer and dissolution of granular follicular pigment into developing oocytes and that mantle pigment was derived from dissolved pigment in the yolk platelets by simply a condensation into granules during development. No chemical transformations were envisioned during these exchanges, merely the mechanical transferring of pigment to and from the yolk platelets.

The present studies have determined, however, that egg and embryo pigment is chemically dissimliar from that found in the mantle of adults. The solubilized pigment in yolk platelets is carotenoid. It is soluble in hydrophobic solvents, including petroleum ether, and has characteristic absorption properties for these compounds (Fox, 1953; Needham, 1974). On the other hand, while the precise chemical nature of the mantle pigment could not be determined, it lacks solubility and the absorbence characteristics of carotenoids and is, in general, a more stable material. Extracts of adult pigment remain unchanged even after months of storage at room temperature, are not decolorized with a strong oxidant ($NaNO_2$), and are resistant to boiling and gross changes in pH.

None of the chemical properties of the mantle pigment allow it to be placed among the better known classes of zoochromes (Needham, 1974). Its solubility in organic-aqueous systems, sensitivity to reducing agents (KBH_4) and dialysis against TCA-acetone would suggest that it is a small, polar molecule. It is not fluorescent and shows no Soret band of absorbence and, hence, does not appear to be porphyrin or pterin (Needham, 1974). Also, it does not share solubility or absorbence properties of bilichromes (Fox, 1953; 1972) or ommochromes (Butenandt, Schiedt, Bierket and Kormann, 1954).

Several attempts have been made to establish the chemical nature of the orange pigment found in blood cells of certain ascidian species which may be related to the pigment in *E. turbinata*. For example, Webb (1939) found that pigment in the orange blood cells in *Ascidia mentula* was sensitive to strong alkaline conditions but could be extracted in mineral acids. The pigment was soluble in water and acetone and resistant to oxidizing agents. Endean (1960) has also analyzed the yellow to orange pigment in blood cells of *Phallusia* (or *Ascidia;* see Webb, 1939) *mammillata.* It was resistant to strong acids, alcohol, and acetone but sensitive to 1 N NaOH. A mixture of $KMnO_4$ and H_2SO_4 also decolorized the pigment bodies, and they gave a positive argentaffin reaction. It was concluded from these observations that the pigment was a melanin.

The orange pigment in blood cells of these two species, then, has some common chemical features. It is resistant to most solvents, but sensitive to alkaline treatment. The yellow mantle pigment in *E. turbinata* is also stable and not soluble in common organic solvents. However, it is not decolorized in 1 N NaOH and, thus, does not share this property with the pigment in the *Ascidia* species. The investigations with these species, however, were largely histochemical and made by observing the pigmented blood cells as the reagents being tested were applied directly to them. The small amount of material in the blood cells precluded extracting the pigment for more thorough, large scale, testing. It may be possible, then, that the orange pigment in the blood cells of the *Ascidia* species and in the mantle of *E. turbinata* are similar or related compounds but react differently to alkaline conditions. If the orange pigment in blood cells of the *Ascidia* species is indeed melanin (Endean, 1960), then it would appear that the mantle pigment in *E. turbinata* is not related to it. It does not have the absorbence characteristics of eumelanin (Needham, 1974) and does not change from yellow to pink in alkaline *vs.* acid conditions, as is the case for erythromelanin (Fox and Vevers, 1960). Further chemical analyses will be needed to determine the constituent elements and structure of this unusual animal pigment.

Some physical characteristics of pigment bearing corpuscles in blood cells of *A. mentula* and *A. mammillata* have also been examined. They exhibit strong birefringence, which is not completely extinguished under any conditions (Webb, 1939; Endean, 1960). Ultrastructurally the pigment bodies in *A. mammillata* are composed of flat, intercalated disks oriented lengthwise along the long axis of the organelle (Endean, 1960). In both the *Ascidia* species, then, the orange pigment bodies are elliptical structures apparently comprised of regularly arranged paracrystalline subunits.

The bodies of *E. turbinata* were also found to have a regular subunit structure, comprised of tubes running parallel to the long axis of the granule. Woollacott (1974) discovered that the pigment vesicles in the eye spot of *Ciona intestinalis* have tubular subunits, but in this case the tubes have no particular orientation and are entwined and enmeshed into compact vesicles. The pigment vesicles in this species are obviously localized in cells.

The individual stellate masses of pigment bodies in *E. turbinata* may originate from single cells or perhaps groups of cells. Bodies resembling a nucleus in size and ultrastructure were found in association with clumps of pigment bodies (Fig. 2). The pigment bodies, however, were in regions obviously larger than other cells and filled with a matrix of fibrils, apparently enclosed in a cytoplasmic membrane. Common cytoplasmic organelles such as mitochondria, endoplasmic reticulum, or lipid bodies were not seen within the fibrillar matrix. It is possible that a stellate mass of pigment bodies is derived from a cell or group of cells that becomes fixed in the mantle tissue, synthesizes pigment granules that flow into extensive cytoplasmic arms and then degenerates, leaving a nuclear remnant and cytoskeleton of fibrils filled with metabolically inactive pigment bodies. This is purely speculative but does offer some explanation for the difficulties encountered in attempting to demonstrate nuclei in these masses using standard light microscopic techniques (Simkins, 1924).

The function of orange pigment in ascidians is not known (Endean, 1960). It has been speculated that it represented an oxidative state of vanadium and that vanadium was acting as a respiratory enzyme (George, 1926; Baldwin, 1949). These hypotheses have been discounted, however, for the lack of evidence both that the orange pigment contains vanadium and that the presence of vanadocytes enhances oxidative reactions (Webb, 1939). The orange to red pigment in the eye spot of *C. intestinalis* has not been characterized chemically, but the action spectrum for sperm release in this species is indicative of a specific photoreceptor, and the pigment has been implicated in this mechanism (Lambert and Brandt, 1967). Woollacott (1974), however, did not find any connections between the sperm duct and pigment cells in an electron microscopical study of this region and postulated instead that the pigment was acting as a barrier against the wavelengths that triggered sperm release.

The location and distribution of the orange mantle pigment in *E. turbinata* would seem to argue against its possible roles as a photoreceptor or as a respiratory catalyst. Its strong absorbence in the UV region of the spectrum may offer some protection against radiation damage to the exposed tissues at the siphon end. However, any functional role ascribed to the pigment should consider the subjectively similar color of the embryos and adults versus the chemical dissimilarities of their pigments.

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SUMMARY

Some chemical properties of the orange pigment from eggs and embryos and the mantle tissue of adult *Ecteinascidia turbinata*, as well as ultrastructural characteristics of pigment masses in the mantle, have been examined. The diffuse egg and embryo pigment has solubility and absorbence characteristics of carotenoids, which are solubilized within yolk platelets. The granular mantle pigment, however, is not carotenoid. It is soluble only in organic-aqueous systems, stable to boiling and gross changes in pH, and absorbs strongly in the UV region with a single major peak at 360 nm. Mantle pigment is found in pigment bodies which ultrastructurally are composed of apparently straight and continuous tubular subunits, approximately 500 to 600 Å in diameter, and oriented parallel to the long axis of the body. The pigment bodies are not localized in obvious cells but found within large areas of fibrillar material that are bound by cytoplasmic membranes and occasionally contain an apparently degenerate nucleus. It may be that mantle pigment is derived from pigment cells of the blood which lose their typical cellular appearance as they become permanent features of the mantle tissue.

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LARVAL DEVELOPMENT AND HABITS OF *LAEONEREIS CULVERI* (WEBSTER) (POLYCHAETA: NEREIDAE)[1, 2]

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Laeonereis culveri (Webster) is a nereid polychaete which occurs from Connecticut to Florida, the Gulf of Mexico, Central America and the east coast of South America, where it abounds in such diverse habitats as estuaries, salt flats, sandy shoals and muddy tidal flats (Webster, 1879; Hartman, 1951; Behre, 1950; Hedgpeth, 1950; Frankenburg and Burbanck, 1963; Pettibone, 1971).

Although *L. culveri* is widely distributed, the only published account of its reproduction is by Klesch (1970) who studied a population in Texas. He found *L. culveri* to be atokous and to undergo embryonic development directly into a 3-setiger larva. He was unable, however, to rear or describe larvae beyond the 3-setiger stage and did not study larval habits.

The present account describes larval development and habits of *L. culveri* from Connecticut. It includes details on early development additional to those provided by Klesch (1970), as well as a description of later development to the adult. An attempt is made to relate observations in this study to the habitat of *L. culveri* and to the array of reproductive patterns exhibited by nereids (Reish, 1957; Clark, 1961).

MATERIALS AND METHODS

This study is based on a population of *L. culveri* inhabiting the Mystic River Estuary in eastern Connecticut. The estuary flows in a southerly direction, opening into Fishers Island Sound at 41° 19′ N, 71° 58′ W (U. S. Coast and Geodetic Chart No. 358). *Laeonereis culveri* is present at the head and upper reaches of the estuary, in the Pequotsepos tributary, and in a small cove on the western shore opposite the Mystic Seaport. The latter cove was chosen as the field site for this study since it is readily accessible and is almost completely exposed as a tidal flat at low tide. Here *L. culveri* occurs abundantly in soft sediments where individuals occupy rust-colored, mucous-lined burrows. In common with other sites populated in the estuary by *L. culveri,* the cove is characterized by extreme diurnal as well as seasonal fluctuations in salinities and temperatures. For instance, at the entrance-way to the cove, temperatures and salinities may range seasonally from 0–30° C and from < 0.5–30 ‰, respectively. These values are based on measurements taken aperiodically at different seasons and various stages of the tide from

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[2] Contribution No. 103 from the Marine Research Laboratory, University of Connecticut, Noank and No. 79 from the Ira C. Darling Center, University of Maine, Walpole.

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July, 1965 to August, 1967; temperatures were measured with a glass bulb thermometer and salinities were determined either by Barnes' (1957) method of titration with $AgNO_3$ or with a hydrometer. The broad fluctuations in salinity within the cove reflect seasonal variations in fresh water discharge from an entering stream.

Adults were readily collected from sediments taken by spade, to a depth of 10 cm, and washed with sea water on a 500 μ screen. Larvae were extracted from sediments skimmed off the tidal flat to a depth of about 2 cm, washed on a 105 μ screen, and then backwashed into a plastic dish for examination under a binocular dissecting microscope. By these methods, the population was qualitatively sampled at least once each month for the seasonal occurrence of larvae and sexually mature adults. The samples also provided worms for laboratory cultures and descriptive purposes.

Unless otherwise noted, all laboratory cultures were maintained at 22 ± 3° C and in sea water with a salinity of about 30‰. The sea water was filtered, prior to use, through a Millipore prefilter pad (Millipore Filter Corporation, Bedford, Massachusetts).

Gamete-bearing worms were individually isolated in 100 cc finger bowls partially filled with sea water, but without sediments. The green alga, *Enteromorpha,* was dried, ground into a coarse powder, and introduced to the finger bowls as food (Reish and Richards, 1966). Worms established mucoid tubes along the walls of the finger bowls and readily ingested the *Enteromorpha.* Every three to four days, worms were transferred to new containers and fed a small amount of powdered *Enteromorpha.* They were also checked periodically for their state of maturity. Although they reproduced in the atokal condition, sexually mature adults were easily distinguished from immature ones by body color, the latter being brown while the former were green (Klesch, 1970). Mature oocytes were readily seen in the coeloms of females while mature males had a creamy appearance due to an abundance of sperm. Worms bearing immature gametes were present in the tidal cove from February to November, while individuals with mature gametes were available from May to October.

Neither males nor females could be induced to spawn in the presence of the opposite sex or of gametes from either sex. Males shed sperm when prodded and gently agitated, but all attempts to induce females to spawn failed. However, females spawned spontaneously, though unpredictably, within four weeks after being collected and kept at 22 ± 3° C. Maintaining females at 14 ± 1° C prevented them from spawning for up to ten months after being collected. In this way, mature females were occasionally held until there was a need for spawned eggs, at which time they were placed at 22 ± 3° C and spawned within two weeks. Ripe males were similarly manipulated to provide sperm when needed.

Successful fertilizations were seldom attained when sperm were mixed with eggs obtained by rupturing the body of a female—even if the eggs were first washed on a fine nylon screen to remove coelomic contaminants. On the other hand, successful fertilizations occurred much more frequently when naturally spawned eggs were mixed with sperm. Mature females, therefore, were examined daily for the presence of spontaneously spawned eggs. The eggs were removed by pipette to a finger bowl containing about 300 cc of sea water. Sperm were obtained by gently prodding a male with forceps. Thus agitated, the male would swim rapidly, lashing the rear of the body from side to side and shedding a cloudy stream of sperm from the pygidium. Three drops of sperm suspension were mixed with the eggs in the finger bowl. Time of fertilization was considered to be the time at which eggs and sperm were mixed. At 15–20 minutes after fertilization, the water was partially decanted from the finger bowl and replenished. This procedure was repeated several times to eliminate surplus sperm. Water was then changed daily until the 3-setiger larval stage had fully developed. The larvae then were removed in groups of 50 to Stender dishes containing sediment about 5 mm in depth. The sediment came either from the cove or from a nearby tidal flat and consisted of fine, flocculent particles rich in benthic diatoms which served as food. For two to three weeks prior to use in cultures, the sediment was stored in finger bowls containing Miquel's sea water medium (Needham, Galtsoff, Lutz and Welch, 1937) and placed under subdued illumination at 22 ± 3° C. This treatment resulted in an increased production of benthic diatoms. Potential predators, larvae, and juveniles of *L. culveri* from the natural population were removed before adding the sediment to cultures. Every three or four days the water was removed by pipette from each larval culture and new water added. Every six or seven days, a small amount of additional sediment was pipetted into the culture.

Larval responses to various substrates were studied by using 5 cm funnels. The stems were removed from the funnels and the bases of the funnels were closed by fusing the glass with an acetylene torch. In use, a funnel was filled with sea water and the sediment to be tested was added to a depth of about 2 cm. Larvae were then added, and the funnel covered with a watch glass. The number of larvae used and the characteristics of the sediments tested are decribed later. All tests were conducted at 22 ± 3° C and 30‰. The sloping walls of the funnels facilitated the observation of swimming larvae at all levels of the water column. Since a relatively small amount of sediment could be concentrated to an appropriate thickness in the bottom of the funnel, the time required to search for individuals in the sediments was minimized.

Developmental stages were examined either in hanging drops or under Saran Wrap coverslips (Dean and Hatfield, 1963). Individuals were photographed with a Polaroid camera attached to a phase contrast microscope (Dean, 1963). Outline drawings were made from photographs with the aid of a camera lucida; details were furnished from descriptions and sketches.

OBSERVATIONS AND RESULTS

Embryogenesis and early larval development

As noted by Klesch (1970), mature sperm of *L. culveri* is of a generalized type (Franzen, 1956) and the spherical pale yellow ova are demersal. At a salinity of 30‰, the latter range from 135–162 μ in diameter with a mean diameter of 147 μ.

A fertilization membrane is elevated five to ten minutes after eggs and sperm are mixed. No jelly layer is exuded. The germinal vesicle disappears and both polar bodies are extruded 10 to 15 minutes after fertilization. The first cleavage is unequal and occurs within two hours, followed by the four-cell stage at three hours and the eight-cell stage at four hours. Cleavage is spiral, and in eight to ten hours a cap of colorless transparent micromeres develops over four macromeres.

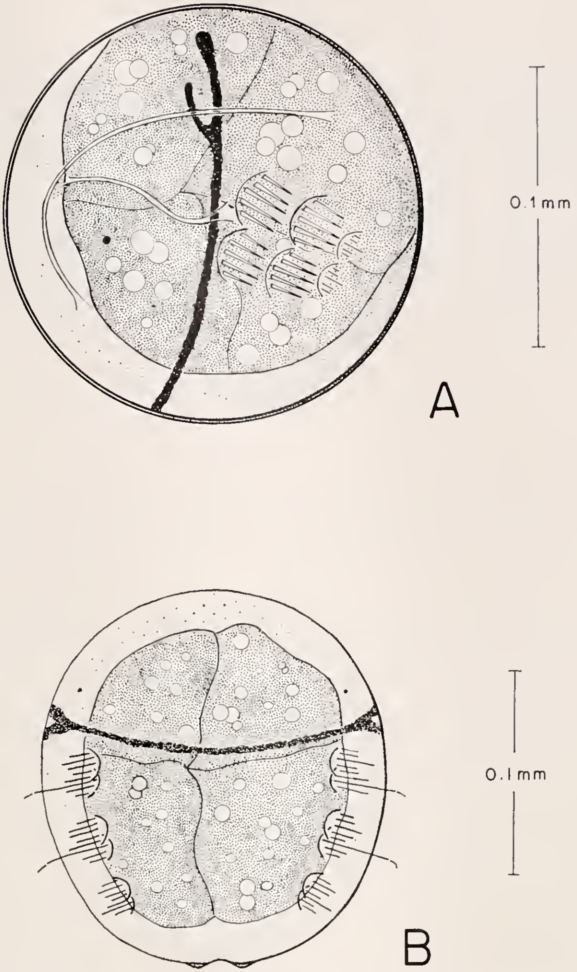

FIGURE 1. Late embryonic stages of *Laeonereis culveri:* (A) forty-eight hour, spherical, setigerous embryo in lateral view; (B) fifty-two hour, elongating embryo in ventral view.

The macromeres are yellow to amber in color and contain most of the yolk. They are unequal in size, the anterior pair being smaller than the posterior pair and the left member of the posterior pair being somewhat larger than the right one. Four macromeres usually occur, but in exceptional cases five or six may form. Embryos with more than four macromeres appear to develop normally. The micromeres completely overgrow the macromeres in typical epibolic gastrulation within 24 to 30 hours.

Further development leads directly to a spherical, setigerous embryo by 48 hours (Fig. 1A). The embryo lacks cilia and does not rotate, but does twitch sporadically. The fertilization membrane and the surface of the body are in loose contact. With each twitch, the body surface is deformed and slightly removed in places from the encompassing fertilization membrane.

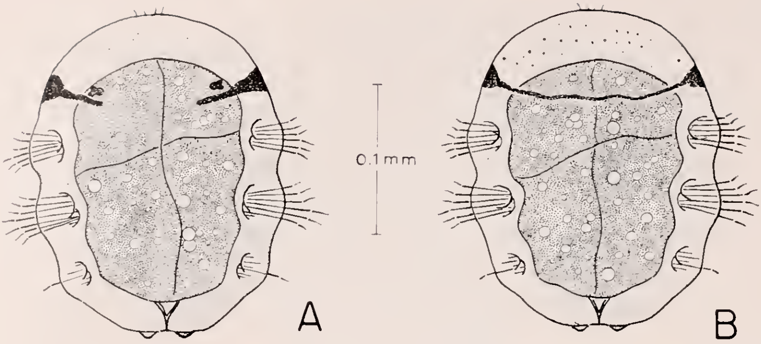

FIGURE 2. Sixty-four hour, early 3-setiger larva of *Laeonereis culveri:* (A) dorsal view; (B) ventral view.

Three pairs of noto- and neuropodial setal sacs develop almost simultaneously in the setigerous embryo. The third pair of setal sacs appears to lag slightly behind the first two pairs in its development. Each setal sac contains five or six simple setae.

A bright red, coarsely granular band of pigment encircles the equator of the spherical, setigerous embryo except for a broad mid-dorsal gap. The equatorial band of pigment divides the body into a setigerous trunk region and an asetigerous head region. All or part of this band may occasionally be absent. Two red pigment spots are generally present on the ventrolateral surface of the head region.

The embryo begins to elongate within 52–60 hours (Fig. 1B). Twitches occur frequently, and each twitch results in a sudden lateral compression of the body. The fertilization membrane is now firmly attached as a cuticle to the outer surface of the body wall. A pair of papillae, anlagen of the anal cirri, project from the posterior extremity of the body.

As the embryo continues to elongate, each simple internal seta is transformed into a compound homogomphous seta consisting of a shaft and serrated blade. Setal shafts then grow outward so that the blades and parts of the shafts protrude through the cuticle. Concurrently, rudimentary parapodial lobes form and externally delimit the three trunk segments which correspond to the three pairs of setal sacs. The first two pairs of parapodia develop more rapidly than the third pair.

At 64 hours (Fig. 2A, B), all three pairs of parapodial lobes have formed, most of the setae protrude through the cuticle, and the body now consists of a massive head and three trunk segments. This stage of development is arbitrarily designated as an "early" 3-setiger larva. Further development leads to a "mid" 3-setiger larva by the third day (Fig. 3), followed by a "late" 3-setiger larva on the fourth day (comparable to the 5-day-old larva shown in Fig. 4A, B). The 3-setiger larval stage persists until about the seventh day, when a rudimentary fourth setiger appears.

In the early 3-setiger larva, the equatorial band of pigment delimits the approximate posterior boundary of the head, which at this stage is not externally

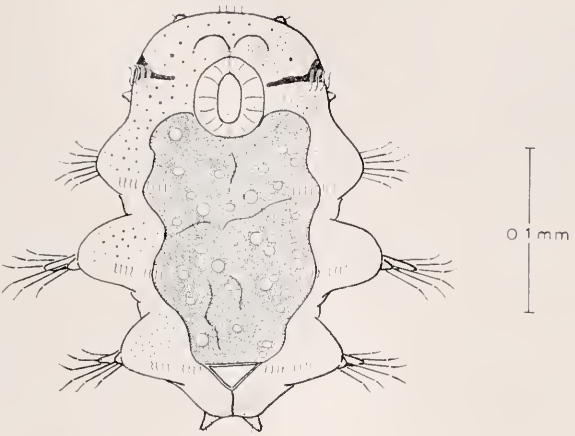

FIGURE 3. Three-day, mid 3-setiger larva of *Laeonereis culveri* in ventral view.

demarcated from the trunk. The head elongates and becomes sharply demarcated from the trunk in the late 3-setiger larva.

As the head elongates, the equatorial pigment-band and the pigmented spots are further removed from the trunk region. A ventral median gap appears in the equatorial pigment-band of the mid 3-setiger larva. The remnants of the equatorial band are gradually concentrated into a pair of lateral pigment patches in the late 3-setiger larva. Each patch is bilobed, consisting of a large dorsolateral lobe and a smaller ventrolateral lobe. The pigmented spots assume ventral positions and come to lie anterior to the labial palps of the late 3-setiger larva.

The simple parapodial lobes of the early 3-setiger larva elongate rapidly, and in the mid 3-setiger stage, each parapodium divides into noto- and neuroaciculate lobes. In addition, an elongated digitate lobe appears midway between the acicular lobes of the second and third parapodia.

Two pairs of dark red eyes develop on the dorsal surface of the prostomium of the early 3-setiger larva. The median pair of eyes is doughnut-shaped and each median eye lies in contact with a lateral cup-shaped eye.

Rudimentary cephalic appendages are present in the mid 3-setiger larva and include a pair of frontal antennae, a pair of ventrally directed labial palps, and a pair of tentacular cirri. All of these appendages elongate to become prominent in the late 3-setiger larva.

A broad suboval pygidium is distinctly delimited from the third trunk segment in the mid 3-setiger larva, and the anal cirri continue to lengthen.

The cephalic appendages and anal cirri are clothed with sensory hairs. Sensory hairs also project from the anterior margin of the prostomium.

A large oval pharynx with a well defined lumen is visible in the mid 3-setiger larva. The pharynx projects posteriorly into the first trunk segment and houses a pair of simple jaws in the four-day-old, late 3-setiger larva. At this stage, each jaw bears a terminal and a basal tooth. On the fifth day, a weakly sclerotized tooth forms midway between the terminal and basal teeth. Horizontal abductor-adductor movements of the jaws were first observed in the 3-setiger larva on the sixth day of

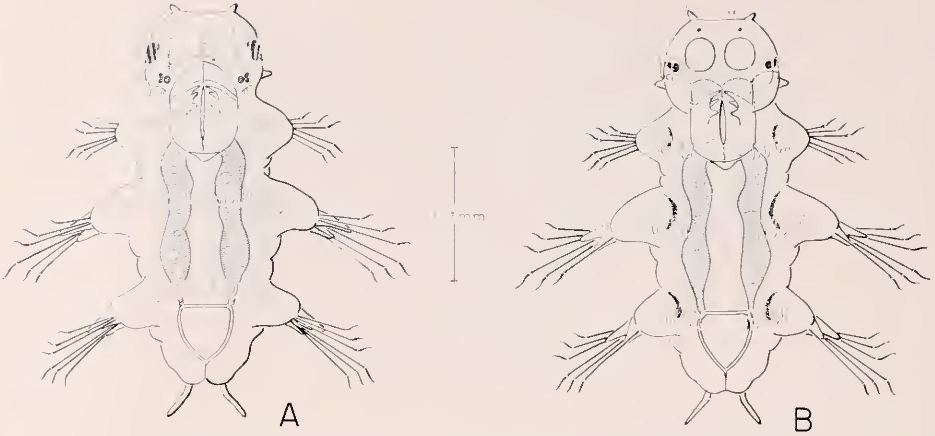

FIGURE 4. Five-day, late 3-setiger larva of *Laeonereis culveri:* (A) dorsal view; (B) ventral view.

development. At this time, the proboscis (pharynx) exhibits weak protractor-retractor movements. Movements of the jaws and proboscis coincide with the onset of feeding.

The midgut of the early 3-setiger larva is massive and projects into the prostomium. The interior of the midgut is filled with the yolky macromeres. Gradually, the macromeres are digested and broken down to an amorphous mass of yolk that flows back and forth within the midgut of the four-day-old, late 3-setiger larva. In the five-day-old larva, the yolk is reduced to two residual masses along the wall of the midgut. A lumen occurs medially between these yolk masses, and yolk droplets flow back and forth within the lumen. On the sixth day of development, the larva feeds actively and the midgut is devoid of yolk except for an occasional yolk droplet mixed with ingested food.

The anlage of the esophagus occurs in the late 3-setiger larva as a plug-like mass of tissue projecting from the pharynx into the anterior portion of the midgut lumen.

The hindgut forms as a small blind sac posterior to the midgut of the early 3-setiger larva. The hindgut lengthens, broadens ,and becomes continuous with the midgut in the six-day-old larva.

Weak ciliary tracts form on the mid 3-setiger larva. The cilia elongate and the tracts become prominent in the late 3-setiger larva. A prototroch encircles the prostomium except for a broad dorsomedial gap. The prototroch consists of two lateral and four ventral patches of cilia.

The prostomium also bears a short patch of cilia located posterolaterad of each set of dorsal eyes (see Fig. 4A). Each ciliary patch lies within a very shallow depression which in later development deepens and constitutes the ciliated nuchal organ.

Nototrochs and gastrotrochs are present on the trunk segments. Each nototroch consists of eight patches of cilia that extend between the bases of parapodia as a dorsal transverse tract. The gastrotrochs consist of two ventral patches of cilia which extend transversely across the base of each parapodium.

The early 3-setiger larva continues the characteristic twitching movements first observed in the elongating embryo. As the parapodial lobes continue to lengthen, the twitches occur more frequently. With each twitch, the parapodia paddle back and forth; however, the larva is incapable of locomotion and remains in one position on the substrate. Likewise, the mid 3-setiger larva remains *in situ* although its body can move from side to side. Upon completion of ciliature development on the fourth day, the 3-setiger larva readily swims and crawls.

Later larval development

A fourth setiger is fully developed at eight days. Beyond this stage, growth becomes increasingly variable. In the presentation which follows, developmental changes are noted without reference to age, and the rate of growth is discussed in a later section. Also, details regarding setal and parapodial development are omitted to await later publication.

Tentacular cirri and peristomium. Four pairs of tentacular cirri eventually develop and are positioned with respect to one another, anterodorsally, posterodorsally, anteroventrally, and posteroventrally.

Tentacular cirri of the 3-setiger larva represent the definitive anterodorsal tentacular cirri. The anteroventral tentacular cirri form as budlike rudiments beneath the anterodorsal cirri in the 5-setiger larva.

The first parapodium of the 5-setiger larva acquires a medial digitate lobe which elongates considerably as the larva advances to a 7-setiger stage (Fig. 5). Upon development of the eighth trunk segment, the first parapodium is reduced to an asetigerous base supporting the elongated digitate lobe, which is now established as the posterodorsal tentacular cirrus (Fig. 6). A budlike rudiment of the posteroventral tentacular cirrus is also present. Cephalization of the first trunk segment is now complete, the latter having been incorporated into the peristomium, which bears the four pairs of tentacular cirri. Since the definitive postlarval form is established, the worm at this stage may be termed a 7-setiger juvenile as distinguished from the preceding 7-setiger larval stage.

The tentacular cirri elongate but at different rates. The posterodorsal tentacular cirri exhibit the most rapid growth and become the longest tentacular cirri in the 8-setiger juvenile. In the 20-setiger juvenile, the relative lengths of the tentacular cirri are proportionately the same as in the adult.

Ciliature. The prototroch is reduced to a few tufts of lateral cilia on the 7-setiger juvenile (Fig. 6) and is absent from the 8-setiger juvenile.

Remnants of the gastrotrochs remain on the 5-setiger larva and are absent from the 6-setiger larva.

A nototroch is acquired on each trunk segment which forms (Fig. 5), up to and including the 7-setiger juvenile stage. The nototrochal cilia are of equivalent lengths on the first four trunk segments but are progressively shorter on the fifth through eighth trunk segments. After the first trunk segment is cephalized, the first nototroch remains as a transverse ciliary band extending between the bases of the posterodorsal tentacular cirri. All nototrochs are present until the juvenile has acquired 16–18 setigers.

The ciliated nuchal organs are retained throughout the life of the worm.

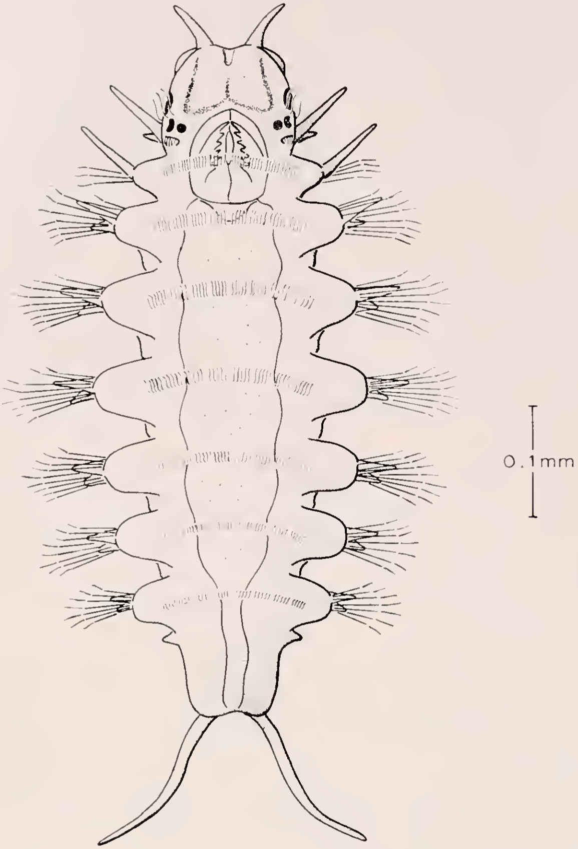

Figure 5. Seven-setiger larva of *Laconereis culveri* in dorsal view.

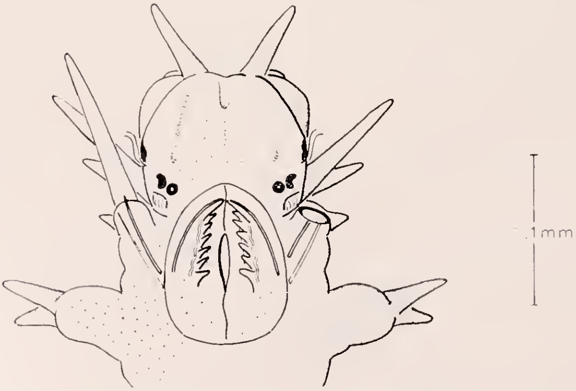

Figure 6. Head and first metastomial segment of a 7-setiger juvenile *Laconereis culveri* in dorsal view; right posterodorsal tentacular cirrus removed; setae and nototroch omitted.

TABLE I

Number of teeth present on each jaw piece during different stages in the life history of Laeonereis culveri *(based on five to ten individuals examined in each life history stage).*

Life history stage (Number of setigers)	Number of teeth (Minimum and maximum)
3 (late stage, 5–6 days old)	3
4	4
5	5
6–7 setigers (7 segments)	5–6
7 setigers (8 segments)	6–7
8–12	7–8
13–14	8–9
15–16	9–10
17–29	10–11
30	11–12
Adult	12–18

Pigmentation. The red pigmented patches and spots on the prostomium gradually diminish in size; they are usually absent from the 8-setiger juvenile but may occasionally persist up to the 12-setiger stage.

Additional pigmentation forms on the five-day-old 3-setiger larva. The pigmentation appears pale yellow to white in incident light, but is black in transmitted light. There is a W-shaped band on the dorsal surface of the head and crescent-shaped bands ventrally on the bases of the first three pairs of parapodia (Fig. 4A, B). The latter bands are most prominent on the second and third pairs of parapodia. The W-shaped band is occasionally incomplete or lacking, but the crescent-shaped bands are invariably present.

The crescent-shaped bands of the first parapodia become greatly reduced in the 6-setiger larva and are absent from the 7-setiger juvenile upon cephalization of the first trunk segment. The remaining crescent-shaped bands are found up to the 15-setiger stage, while the W-shaped band may persist up to the 17-setiger stage.

Jaws and alimentary tract. Jaw development up to the 5-setiger larval stage is relatively uniform (Table I). The number of teeth present on the jaws in subsequent life history stages varies; and the definitive number of teeth is attained at about the 30-setiger stage.

The pharynx, when retracted, occupies the first trunk segment in 3- to 7-setiger larvae. The pharynx expands and elongates during later development and eventually occupies the first four metastomial segments of the adult. The pharynx of *L. culveri* is characterized by the presence of tufts of fleshy papillae instead of paragnaths. The 20-setiger stage was the earliest stage in which the papillae were observed.

The esophagus remains as a plug-like mass projecting into the anterior end of the midgut until the 12-setiger stage. During subsequent development, the esophagus elongates until it occupies the fifth to twelfth metastomial segments of the adult, with the posterior end still projecting into the midgut.

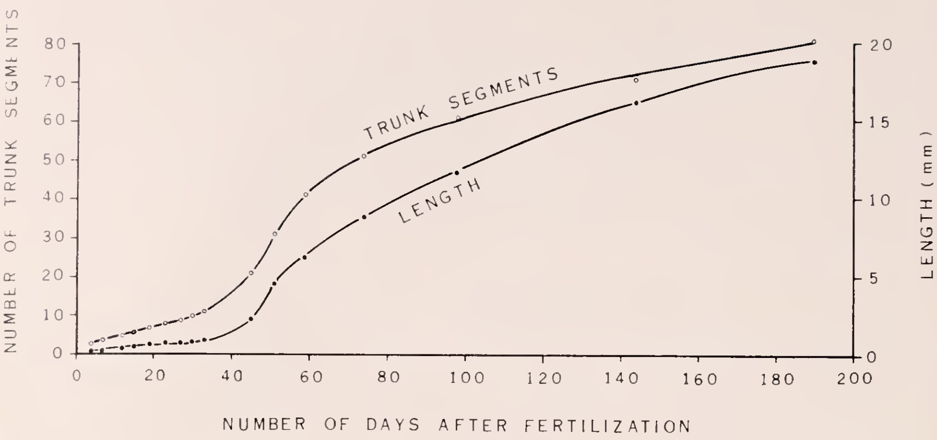

FIGURE 7. Growth of *Laeonereis culveri* in laboratory cultures at 22 ± 3° C and 30‰. Values plotted are means of 12–24 individuals.

In worms with less than 30 setigers, the midgut can be readily distinguished from the hindgut on the basis of pigmentation and general form. The midgut is yellow, relatively broad, and highly expanded; the hindgut is colorless, relatively narrow, highly sinuous, and sometimes doubles back on itself. The hindgut elongates more than the midgut. Thus in the 30-setiger juvenile, the midgut occupies the fifth to twelfth metastomial segments, while the hindgut occupies the 13th to 30th metastomial segments. Beyond the 30-setiger stage, distinction between mid- and hindgut requires histological examination.

Growth under laboratory conditions

As noted previously, the growth of laboratory-reared worms was highly variable beyond the 4-setiger larval stage and individual variation became progressively greater as cultures aged. Unfortunately, the culture techniques which were used did not allow for careful control of the quality and quantity of food. Therefore, it is impossible to judge if the variability in growth was inherent or due to differences in food conditions. Consequently, growth of laboratory-reared worms was followed by noting the earliest appearance of a given life history stage and its corresponding length (Table II). A sigmoid growth curve resulted when either length or segment-formation was used as an indicator of growth (Fig. 7).

Thirty-two specimens (17 females, 15 males) of the F_1 generation were successfully reared to sexual maturity and spawned after 168–192 days of development. The F_2 generation of worms appeared identical in morphology to those of the F_1 generation.

Sexually mature, laboratory-reared worms ranged from 16.5–20.0 mm in length, in contrast to those from the natural population, which ranged from 28–45 mm in length. The lengths were taken from worms relaxed in a 0.15% solution of propylene phenoxytol in sea water.

TABLE II

Development and growth of Laeonereis culveri *reared in the laboratory at 22 ± 3° C and 30‰. All lengths are mean values calculated from measurements of one to two dozen specimens. Postembryonic stages were relaxed in 0.15% propylene phenoxytol—sea water solution prior to being measured. Age is time after fertilization.*

Stage of development	Length (mm)	Age
Fertilization membrane elevated	—	5 minutes
Both polar bodies extruded	—	10 minutes
2-cell stage	—	1.5 hours
4-cell stage	—	3.0 hours
8-cell stage	—	4.0 hours
Early gastrula	—	8.0 hours
Late gastrula	—	24 hours
Spherical setigerous embryo	0.15	2 days
3-setiger larva (free-moving)	0.27	4 days
4-setiger larva	0.35	8 days
5-setiger larva	0.42	12 days
6-setiger larva	0.51	15 days
7-setiger larva	0.59	19 days
7-setiger postlarval stage	0.65	23 days
8-setiger postlarval stage	0.70	27 days
9-setiger postlarval stage	0.76	30 days
10-setiger postlarval stage	0.85	33 days
20-setiger postlarval stage	2.30	45 days
30-setiger postlarval stage	4.60	51 days
40-setiger postlarval stage	6.40	59 days
50-setiger postlarval stage	8.70	74 days
60-setiger postlarval stage	11.70	98 days
70-setiger postlarval stage	16.50	144 days
80-setiger postlarval stage	18.80	190 days

Benthic mode of existence

Neither eggs nor larvae were ever encountered in numerous plankton samples taken aperiodically from May to October (in 1966 and 1967) at the entranceway to the cove where *L. culveri* was very abundant. However, larvae were plentiful in all stages of development, from June to September, in the uppermost 2 cm layer of flocculent sediment on the tidal flats of the cove. Therefore, it is evident that *L. culveri* undergoes a benthic development. Further evidence supporting this view comes from laboratory observations on spawning and experiments on larval responses to sediments as detailed below.

All females which spawned in the laboratory, deposited eggs within their mucoid tubes. The spent female always remained in the tube which was irrigated by rapid dorsoventral undulations of the female's body that created a unidirectional water current and brought in sperm, whenever they were added to the water. Subsequently, the irrigational currents served to aerate the embryos until they developed to 3-setiger larvae which either crawled or swam out of the maternal tube.

Interestingly, it was only with the greatest difficulty that a spent female could be prodded out of her tube; spent males, however, were easily forced to swim out of their tubes after being prodded lightly. It was also observed that spent individuals of same or opposite sex could dwell in harmony in the same tube together

TABLE III

The effect of various sediments on the burrowing response of four-day-old 3-setiger larvae of Laeonereis culveri *(experiments terminated and results taken after a period of 12–20 hours; 50 larvae used per experiment).*

Sediment used	Number of experiments	Mean percent swimming-crawling	Mean percent burrowed in sediment	Mean percent mortality
Fine, flocculent sediment	10	2.0	97.0	1.0
Very fine to fine sands, particle diameters, 63–250 μ	5	2.8	95.6	1.6
Medium sands, particle diameters, 250–500 μ	4	95.0	4.0	1.0
Very coarse sands, particle diameters, 1000–2000 μ	5	78.8	17.2	4.0

with spawned eggs or embryos. Fighting responses or attempts to evict other occupants were never observed; indeed all life history stages appeared nonagressive.

Klesch (1970) found that spent worms die 24 hours after spawning, but in the present study they were observed to live for 10 to 16 days after spawning. Further, they continued to ingest food until one to two days prior to death. Occasionally a spent female even ingested a few eggs or embryos; the latter passed intact through the alimentary canal but became entrapped in mucoid fecal strings and decomposed. In any event, the duration of life after spawning was more than sufficient to permit the embryos to develop to freely moving 3-setiger larvae. Apparently in the natural habitat, developing eggs occur in maternal burrows and the 3-setiger larvae subsequently migrate to the surface layers of sediment. This would account for the observed absence of eggs and embryos, but the presence of larvae, in the upper 2 cm of sediments in the cove.

Whenever 3-setiger larvae were placed in a dish without sediment, they alternately swam and crawled. When light from a microscope lamp was transmitted up through the bottom of the dish, the larvae swam and crawled along the bottom. If the same light source was positioned above the surface of the water and to one side of the dish, the larvae would swim up toward the light. However, at various intervals, individual larvae returned to the bottom where they crawled toward the more shaded portions of the dish. Several minutes of crawling were again followed by swimming, and the larvae continued to alternately swim and crawl.

If an appropriate sediment was available, 3-setiger larvae rapidly entered the sediment where they formed delicate mucous-lined burrows and ceased to exhibit the swim-crawl behavior. Not all sediments elicited the burrowing response. Larvae continued to swim and crawl in the presence of medium to very coarse sands which ranged from 250–1000 μ in particle diameter (Table III). Occasionally, individuals entered interstices between the sand grains but soon emerged and resumed swimming and crawling. Larvae readily burrowed into sediments consisting of particles finer than 250 μ in diameter.

If the fine-particled, "attractive" sediments were either air-dried or ashed prior to being offered to larvae, as strong a burrowing response occurred as that elicited by the untreated sediment (Table IV). This suggests that the presence

TABLE IV

The effect of two treatments of the flocculent, surface sediment from the natural habitat (tidal flat) on the burrowing response of five-day-old 3-setiger larvae of Laeonereis culveri *(experiments terminated and results taken after 12–16 hours; 50 larvae used per experiment).*

Treatment of sediment	Number of experiments	Mean percent swimming-crawling	Mean percent burrowed in sediment	Mean percent mortality
Untreated	4	2.5	95.5	2.0
Washed with distilled water and ashed 500° C for four hours	4	1.0	97.5	1.5
Washed with distilled water and air-dried	5	2.8	95.0	2.2

of organic films, detritus, or living food material (*e.g.,* benthic diatoms) is not required to elicit the burrowing response. The primary factor governing the burrowing response of larvae is the particle-size composition of the sediment.

As long as environmental conditions are suitable, larvae remain within their vertical burrows where they constantly move up and down. Partial emergence from the burrows occasionally occurs, followed by rapid withdrawal. While partially emerged, a larva may be observed to engulf benthic diatoms on the surface of the sediment. On one occasion, an attempt was made to feed powdered *Enteromorpha* to a culture of 3-setiger larvae residing in sediment. An excessive amount of the food was added and it soon decayed. The larvae then emerged from their burrows and swam to the surface of the water.

Four- and 5-setiger larvae retain the ability to swim by means of cilia, and their behavioral responses are identical to those of 3-setiger larvae. The cilia no longer function in swimming once the sixth setiger is formed, and later developmental stages can swim only for short distances by lateral undulations of the body. Although nototrochs do not disappear until the juvenile has acquired 16–18 setigers, these ciliary tracts function solely to create a ventilation current through the burrow and are much too weak to propel the juvenile up into the water column. However, the ventilation current is strong enough to flush fine particles of sediment and diatoms through the burrow. With loss of the nototrochs, the worm produces ventilation currents by dorsoventral undulations of the body.

Feeding habits

The gut contents of numerous specimens from the natural populations were examined. They revealed that 3- to 5-setiger larvae almost exclusively ingest benthic diatoms while older life-history stages ingest sediment and detritus.

The uppermost, flocculent layer of sediment where larvae reside on tidal flats contains an abundance of benthic diatoms. Unless larvae were provided with either fresh flocculent sediment or with sediments enriched with benthic diatoms in Miquel's medium, growth and development in the laboratory beyond the 3-setiger stage was impossible. It was also noted that if the flocculent sediment was air-dried, prior to use in larval culture, growth and survival were negligible (Table V). These observations suggest that although organic detritus may serve as food for 3- to 5-setiger larvae, it is a poor substitute for living benthic diatoms. Several

TABLE V

The effect of various substrates on survival and early growth of four-day-old 3-setiger larvae of Laeonereis culveri *after a period of eight days.*

Experimental conditions	Number of 4-setiger larvae	Number of 5-setiger larvae	Number of larvae dead	Total number
Flocculent surface sediment from natural habitat (tidal flat); untreated; 2 cm deep	23	23	4	50
	20	17	13	50
	25	20	5	50
Flocculent surface sediment from natural habitat (tidal flat); washed with distilled water and air-dried; 2 cm deep	6	0	44	50
	5	0	45	50
	10	1	39	50
Flocculent surface sediment from natural habitat (tidal flat); washed with distilled water and ashed for four hours at 500° C; 2 cm deep	1	0	49	50
	0	0	50	50
	2	0	48	50
Benthic diatoms and widely scattered particles of sediment coating glass vessel	0	0	20	20
	0	0	20	20
	1	0	19	20
	1	0	19	20
Phaeodactylum; thin layer; and no sediment	0	0	20	20
	0	0	20	20
	0	0	20	20
Glass culture dish without food or sediment	0	0	20	20
	0	0	20	20
	0	0	20	20
	0	0	20	20

attempts were made to culture larvae on benthic diatoms in the absence of sediment. The diatoms were obtained by placing flocculent sediments in culture dishes for several hours. The sediments were gently washed out, leaving numerous diatoms attached to the inner surfaces of the culture dishes. The 3-setiger larvae which were placed in such dishes fed on the diatoms; however, after a few days the larvae weakened and died. Mortalities were equivalent to those of larvae starved for the same length of time (Table V). Attempts to culture larvae on the diatom *Phaeodactylum* also failed. Thus, growth and survival of 3- to 5-setiger larvae in the laboratory required both the presence of sediment and living benthic diatoms.

Beyond the 7-setiger postlarval stage, powdered *Enteromorpha* was successfully utilized as a food source in cultures when employed with sediment. Upon reaching the 15-setiger juvenile stage, worms could be maintained solely on powdered *Enteromorpha.*

DISCUSSION

Characteristic features of a generalized nereid life cycle include (1) the structural transformation of sexually mature worms from atokal to epitokal forms that swarm near the water surface where they release gametes, and (2) a pelagic

Table VI

Egg sizes and modes of embryonic development of select species of nereids.

Species	Egg diameter (μ)	Ciliated embryo	Earliest larval stage	Reference
Nereis succinea	140–150	Present	Trochophore	Banse, 1954
Nicon aestuariensis	150	Present	Trochophore	Estcourt, 1966
Laeonereis culveri	135–162	Absent	3-setiger	Present study
Platynereis dumerilii	175	Present	Trochophore	Cazaux, 1969
Nereis pelagica	180	Present	Metatrochophore	Wilson, 1932
Nereis virens	170–180	Present	Trochophore	Bass and Brafield, 1972
Nereis grubei	162–380	Present	Metatrochophore	Reish, 1954
Nereis irrorata	210	?	Trochophore	Cazaux, 1969
Nereis fucata	200–250	Present	Metatrochophore	Gilpin-Brown, 1959
Nereis diversicolor	200–275	Present	Trochophore	Dales, 1950
Ceratonereis costae	200	Present	3-setiger	Durchon, 1956
Platynereis massiliensis	250	Absent	3-setiger	Hauenschild, 1951
Perinereis cultrifera	250–400	Present	3-setiger	Herpin, 1925
Nereis arenaceodentata	420–520	Absent	3-setiger	Reish, 1957

larval development, the initial stage being a lecithotrophic trochophore (Reish, 1957; Clark, 1961). However, life cycles of brackish- or freshwater-inhabiting nereids are often modified (Smith, 1958; Clark, 1961). Therefore it isn't surprising that *L. culveri,* which is a markedly euryhaline (Oglesby, 1965), estuarine species (Pettibone, 1971), reproduces in the atokal state and has a benthic larval development in which the trochophore is suppressed.

Elimination of the trochophore from the ontogeny of *L. culveri* is a consequence of the delayed development of locomotory cilia which do not appear until the 3-setiger larval stage. This contrasts with the ontogeny of most nereids in which cilia first form on the gastrula and remain on the trochophore, even if the latter is imprisoned in a jelly layer, *e.g.* as in *Perinereis cultrifera, Perinereis marioni* or *Ceratonereis costae* (Herpin, 1925; Durchon, 1956). The only nereids known to lack ciliated embryos include *L. culveri, Nereis arenaceodentata* (= *caudata*) and *Platynereis massiliensis* which are also unique in having zygotes that never produce jelly layers (Hauenschild, 1951; Reish, 1957); and in each of these species embryogenesis leads directly to a benthic 3-setiger larva, the trochophore being suppressed. The eggs of *N. arenaceodentata* and *P. massiliensis* are, however, extremely large (diameter, 250–450 μ) and give rise to unciliated, lecithotrophic larvae (the nereidogenic larvae of Hempelmann, 1911) which subsist on yolk reserves until late in development, *e.g.,* up to 17- to 19-setiger stages in *N. arenaceodentata* (Reish, 1957). On the other hand, *L. culveri* eggs are relatively small (diameter, 135–162 μ); hence yolk reserves are nearly depleted in the holotrophic 3-setiger larva which, furthermore, has the characteristic ciliary tracts of a planktogenic (Hempelmann, 1911) larva. Thus the retarded development of locomotory cilia and the resultant ontogenetic suppression of the trochophore in *L. culveri* are not associated with the production of very large, yolky eggs. This view is further verified by data in Table VI which show that there are indeed nereids with generalized modes of larval development (having pelagic trochophores or metatrochophores) whose eggs either equal or exceed in diameter those of *L. culveri.*

Although "planktogenic" and capable of swimming, the 3-setiger *L. culveri* larva typically leads a benthic existence in soft, finely particled sediments. Laboratory observations indicate, however, that if water overlying such sediments becomes "foul" (perhaps as a result of extreme organic pollution), the larva will assume a swim-crawl behavior until finding appropriately textured sediments under conditions of favorable water quality—whereupon the larva will reburrow and remain benthic. Presumably, such behavior would also be shown by larvae inadvertently flushed from their burrows by strong tidal currents. In this respect, it's noteworthy that the Berkeleys (1953) observed 3-setiger larvae of *Micronereis nanaimoensis* remaining pelagic for two to three weeks unless encountering a sandy substrate into which they burrowed and left only if agitated. Other "planktogenic" 3-setiger larvae known to be benthic are those of *Ceratonereis costae* (Durchon, 1956) and *Nereis virens* (Bass and Brafield, 1972; personal observations).

Clearly, the possession of planktogenic attributes does not imply that a nectochaetous nereid larva is pelagic. As in *L. culveri,* larval ciliature may function primarily in ventilating burrows and only secondarily in swimming. Indeed, *L. culveri* is apparently unique in having segmental ciliary tracts on each of the first eight trunk-segments and in retaining these tracts for the purpose of burrow ventilation until about the 16-setiger stage when undulatory body movements commence to create ventilating currents. Segmental ciliary tracts of other nereids seldom number more than four, as in *Nereis japonica* (Izuka, 1908) and are lost early in larval life, *e.g.* at the 6-setiger stage of *Nereis fucata* (Gilpin-Brown, 1959).

The reproduction and development of *L. culveri* in Connecticut is generally similar to that reported by Klesch (1970) for the same species in Texas, except for the following details: 1) spawning occurs in Texas from August to September and January to February (temperatures, 15.0–33.9° C; salinities, 28.7–54.0‰); this contrasts with the single spawning season in Connecticut, from June to September (temperatures, 20.5–27.0° C; salinities, 14.8–30.2‰); Connecticut worms were inhibited from spawning in the laboratory at 14 ± 1° C which approximates the minimum seasonal spawning temperature in Texas; 2) unlike in Connecticut, worms in Texas rarely shed gametes spontaneously and fertilization of stripped gametes proved highly successful; 3) Texas worms died 24 hours after spawning (apparently at 22° C; salinity not reported); Connecticut worms survived 10–16 days after spawning (at 22 ± 3° C; 30‰); 4) diameters of mature eggs in Texas *vs.* Connecticut were 150–200 μ and 135–162 μ, respectively; 5) time of development, from fertilized egg to late 3-setiger larva, was seven days in Texas (at 22° C; salinity not reported) but only four days in Connecticut (22 ± 3° C; 30‰); and swim-crawl movements commenced in five-day-old, early 3-setiger Texas larvae but did not occur until the late 3-setiger stage in Connecticut.

The presence of warmer seasonal temperatures in Texas may promote biannual spawning, while in Connecticut spawning is restricted to summer months when temperatures exceed 20° C. Additional study is needed to determine the significance of these as well as other geographic differences in the reproduction and development of *L. culveri.* Further, a geographic comparison of development can presently be made only up to the 3-setiger larval stage since Klesch (1970) did not describe later laval development.

The author is grateful to Dr. David Dean (Director, Ira C. Darling Center, Walpole, Maine) for his invaluable advice and to Dr. John S. Rankin (University of Connecticut Marine Research Laboratory, Noank, Connecticut) for material support during the course of this study.

SUMMARY

The nereid polychaete *Laeonereis culveri* (Webster) reproduces in the atokal condition in the Mystic River Estuary, Connecticut. Females spawn demersal eggs, 135–162 μ in diameter, that do not extrude a jelly layer upon fertilization and that give rise to unciliated embryos. Embryogenesis leads directly to a 3-setiger larva in four days (at $22 \pm 3°$ C; 30‰). The trochophore is suppressed due to the retarded development of locomotory cilia which do not appear until the 3-setiger larva has formed.

Although provided with ciliary tracts and capable of swimming, *L. culveri* larvae are normally benthic in habit. They reside in burrows within the upper 2 cm of fine, flocculent sediments where they feed chiefly on benthic diatoms until developing past the 5-setiger stage after which they become non-selective deposit feeders. The primary function of the larval ciliature is to pass water currents through the burrow. In the laboratory, 3- to 5-setiger larvae alternately swim and crawl in the absence of sediments or in the presence of coarse sediments (particle diameters $> 250\ \mu$); but they readily burrow into and remain within fine sediments (particle diameters $< 250\ \mu$) even if the latter are relatively free of organic matter. Swim-crawl behavior also appears to be elicited under conditions of unfavorable water quality. When the sixth setiger forms, the larva is no longer capable of swimming by means of its cilia.

Development beyond the 3-setiger stage approximates that of other species except that *L. culveri* larvae are apparently unique in ultimately developing eight nototrochs (one per each of the first eight trunk segments). The nototrochs are retained and function in burrow ventilation until the 16- to 18-setiger stages when body undulations commence creating ventilation currents.

Larval development is completed with addition of the eighth trunk segment. At this stage, the tentacular segment (first trunk segment) is incorporated into the peristomium and the first pair of parapodia are modified to form the posterodorsal tentacular cirri.

Sigmoid growth curves resulted when either length or segment-formation was used as an indicator of growth among laboratory-reared worms. Sexually mature, laboratory-reared worms were only about one-half the length of sexually mature worms in a natural population. Thirty-two individuals (17 females, 15 males) of an F_1 generation were reared to maturity and spawned after 168–198 days of development. F_2 generation larvae were identical in morphology to those of the F_1 generation.

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AN ANALYSIS OF SHELL OCCUPATION BY TWO SYMPATRIC SPECIES OF HERMIT CRAB. I. ECOLOGICAL FACTORS

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To protect itself from its environment, a hermit crab must have a gastropod shell (Reese, 1969; Vance, 1972a) and where numbers of gastropod shells are limited, there is evidence that this limitation reduces the number of hermit crabs an environment can support (Hazlett, 1970; Provenzano, 1960; Reese, 1969; Thompson, 1903). In addition to acting as a limiting factor, the type of shells present in any one place can also determine what size or species of hermit crab is present in that area. Reese (1969, p. 346) states that: "in Hawaii the availability of shells seems to limit the size of individuals in the population", while Markham (1968) found that occupation of small shells by the European hermit crab, *Pagurus bernhardus*, restricted the growth of these crabs. Examples of resource partitioning of hermit crab species on the basis of two different types of shell have also been studied. Grant and Ulmer (1974) and Wright (1973) both found that shells covered by *Hydractinia echinata* were preferred by one out of two sympatric species while Vance (1972b) showed that, of the three rocky intertidal species of the San Juan Islands, Washington, one species preferred short light shells whereas the other two species preferred comparatively taller and heavier shells.

In the present study experiments were performed on two species of hermit crabs, *Pagurus longicarpus* and *Clibanarius vittatus*, which were equally abundant in the intertidal zone at Beaufort Harbor, North Carolina. This area had no empty gastropod shells, thus suggesting that shells were a limiting factor in this area and that resource partitioning of the shells may explain the coexistence of the two hermit crab species. The experiments were designed to answer the following questions. (1) Are shells a limiting factor in this area? (2) Does resource partitioning take place between *P. longicarpus* and *C. vittatus?* (3) If resource partitioning does take place, what factors maintain the partitioning?

Materials and Methods

All animals used in this experiment were taken from the intertidal zone of an area approximately 100 meters long at Beaufort Harbor, North Carolina, next to the Duke University Marine Laboratory dock. This area has a mixture of sand and mud as a substrate and has many living gastropods buried in it. All experiments were conducted during the summer months and no difficulty was encountered in obtaining specimens of either species of hermit crab in this area.

Three series of experiments were conducted. The first series tested the effect of shell limitation on the hermit crabs' occupation of shells, while the second tested the ability of one hermit crab species to obtain a preferred species of shell from the other hermit crab species. The third series of experiments tested the relation between substrate preference of both hermit crab species and the natural occurrence of two living gastropod species on different substrates.

Shell selection

Sixty *Pagurus longicarpus* and sixty *Clibanarius vittatus* were collected in shallow water at low tide on 14th July, 1974. The species of shell which each crab inhabited was noted, and for each of these shells, five empty shells of the same species were put into an experimental tank 120 cm × 70 cm × 20 cm deep. This gave a total of twenty-five *Nassarius vibex,* 315 *Ilyanassa obsoleta,* thirty *Urosalpinx cineria,* ten *Thais haemastoma floridana,* 200 *Littorina irrorata* and twenty *Polinices duplicatus.* All shells put into the tank had previously been collected in the nearby area. The ratio of gastropod shells occurring in nature was used rather than using equal numbers of different shell species so that an idea could be obtained of how a hermit crab would select a shell in nature if there was no competition from any other hermit crab for that species of shell.

After all the empty shells had been placed at random in the tank, three of the sixty *P. longicarpus* and three of the sixty *C. vittatus* were evicted from their shells by holding them over a glass plate under which there was a 100 watt light bulb. *P. longicarpus* came out by tugging on the minor cheliped side of its body, while *C. vittatus* climbed out onto my hand after about a minute of gentle heating. This method proved 100% successful and did not seem to harm the crab in any way. After they were evicted, they were blotted dry of excess water, weighed and then placed in the experimental tank where they were left for 12 hours. It did not seem likely that any of the six crabs would choose the same shell, but to make the risk minimal, an effort was made not to put two crabs of less than 0.05 g difference in the tank at the same time.

When the 12-hour period of shell selection had elapsed, the species of shell which each crab was inhabiting was noted and each crab was evicted from its shell and discarded from the experiment. The shells were then placed back in the tank ready for three more crabs of each species to be evicted from their "field" shell and placed in the tank for a 12-hour period. It was not thought likely that the shells chosen by the preceding crabs would have been made more or less attractive by their immediately previous inhabitation, as Jensen (1970) did not find this to be true when testing *Pagurus bernhardus,* and there was no evidence to suggest the contrary in the present experiment. There was also no evidence to suggest that those crabs placed in the tank in the mornings selected shells any differently from those placed in the tank in the evenings, as diffuse artificial lighting was supplied during hours of normal darkness, and no pronounced activity rhythms were apparent in either species.

No feeding, other than the detritus on the sand/mud substrate, was provided in the experimental tank, but in the stock tank, crabs were fed on pieces of meat from the blue crab, *Callinectes sapidus.*

Interspecific shell fights

Large specimens of *P. longicarpus* in *I. obsoleta* shells were collected from the Beaufort Harbor region, evicted from their shells, weighed, and then returned to their shells. Crabs below 0.23 g were rejected from the experiment as the results of the shell selection experiment showed that they may not prefer a *Littorina irrorata* shell to their home shell. *C. vittatus* inhabiting *L. irrorata* shells were then collected from the same area, evicted from their shells to check that none were below 0.23 g, and then the first ten collected were given back their home shells. These ten *C. vittatus,* together with the first ten *P. longicarpus* found to be 0.23 g or over, were then put into the experimental tank, this time with all the empty shells removed, and left for 24 hours. The crabs were then taken out of the tank and note was taken of which species of shell each crab was occupying.

The following day the experiment was repeated, this time using ten *C. vittatus* of 0.23 g or over in *I. obsoleta* shells and ten *P. longicarpus* in *L. irrorata* shells. Owing to the lack of *P. longicarpus* found in *L. irrorata* shells in the field, five of the ten *P. longicarpus* used were given the choice of an *L. irrorata* shell taken at random and their *I. obsoleta* shell in which they were found. In four out of five cases the crabs went into the *L. irrorata* shell and in the other case the crab went into a second *L. irrorata* shell offered to it (the first *L. irrorata* shell was noticeably smaller in size than the *I. obsoleta* shell).

Substrate selection

The substrate preference of *P. longicarpus* and *C. vittatus* for mud or sand was tested by dividing the experimental tank into halves by putting mud to a depth of approximately 2 cm on one half of the tank and coarse sand of an equal depth on the other half. The mud was taken from Bird Shoal, an island very near to Beaufort Harbor, and the sand was taken from Beaufort waterfront, directly across from the Bird Shoal collecting point. These sites were chosen because the former is abundant in living *I. obsoleta,* whereas the latter has many *L. irrorata* sticking to the reeds. No live *L. irrorata* were found at the Bird Shoal site, however, nor was *I. obsoleta* found at Beaufort waterfront. A preference for one substrate over the other by *P. longicarpus* or *C. vittatus* would therefore limit its chance of finding shells commonly occurring on the less preferred substrate.

Three categories of experiments were performed on *P. longicarpus* and *C. vittatus.* These were: (a) isolated crabs; (b) intraspecific groups of ten crabs; and (c) interspecific groups of ten crabs of each species. These categories were used as there is evidence that hermit crab behavior is altered both by isolation (Courchesne and Barlow, 1971; Grant and Ulmer, 1974; Hazlett, 1966; Michell, 1973) and by the presence of another hermit crab species (Meadows and Mitchell, 1973).

The procedure for testing the first category was to put a hermit crab on the sand/mud partition, leave it isolated in the tank for five hours, then note which half of the tank it occupied. A minimum of five hours duration in the tank was chosen, as a hermit crab can be very active in a closed container until its has explored its surroundings. Its activity then drops off markedly. In the present situation, this occurred at a maximum of five hours after being placed in the tank.

In addition to testing differences in the hermit crab species' preferences to mud and sand, it was decided to test if a hermit crab occupying a dark shell, such as *I. obsoleta,* was more likely to occur on mud than one inhabiting a white shell, such as *L. irrorata,* or conversely, if a crab inhabiting *L. irrorata* was more likely to occur on sand than mud. This was done by ensuring that half the crabs of both species tested occupied *I. obsoleta* and the other half occupied *L. irrorata.*

TABLE I

Field collection: number of gastropod shells occupied by each species of hermit crab.

Shell species	*P. longicarpus*	*C. vittatus*
N. vibex	5	0
I. obsoleta	50	13
U. cineria	1	5
L. irrorata	3	37
T. haemostoma floridana	1	1
P. duplicatus	0	4

Finally, after ten crabs of both species were tested, the sand and mud halves of the tank were reversed, and another ten crabs of both species were tested in the same way as before. This reversal of substrates established a control for factors, such as light, which may have influenced the distribution of the crabs when they were in the tank.

The second category of experiments was performed in the same way as the first category, the only difference being that ten crabs of the one species were put into the tank at the same time. Similarly the third category was conducted as in the second category but this time ten crabs of both species were put into the tank at the same time.

RESULTS

Shell selection

The results of the field collection of sixty *P. longicarpus* and sixty *C. vittatus* are shown in Table I.

On analysis by the two-tailed binomial test, the probability that there was no difference between the number of shells inhabited by *P. longicarpus* and *C. vittatus* was < 0.001 for both *I. obsoleta* and *L. irrorata*. Differences in the ratio of the two species occupying other shell species were not significant, or alternatively were not tested owing to the small sample size.

The results of the laboratory shell selection experiment for the same sixty crabs of both species are represented in Table II.

Significant differences in selection of shell species were obtained for *I. obsoleta* and *P. duplicatus* ($P < 0.001$). The only other species with large enough numbers

TABLE II

Laboratory selection: number of gastropod shells selected by each species of hermit crab.

Shell species	*P. longicarpus*	*C. vittatus*
N. vibex	4	0
I. obsoleta	44	4
U. cineria	0	0
L. irrorata	12	18
T. haemastoma floridana	0	5
P. duplicatus	0	33

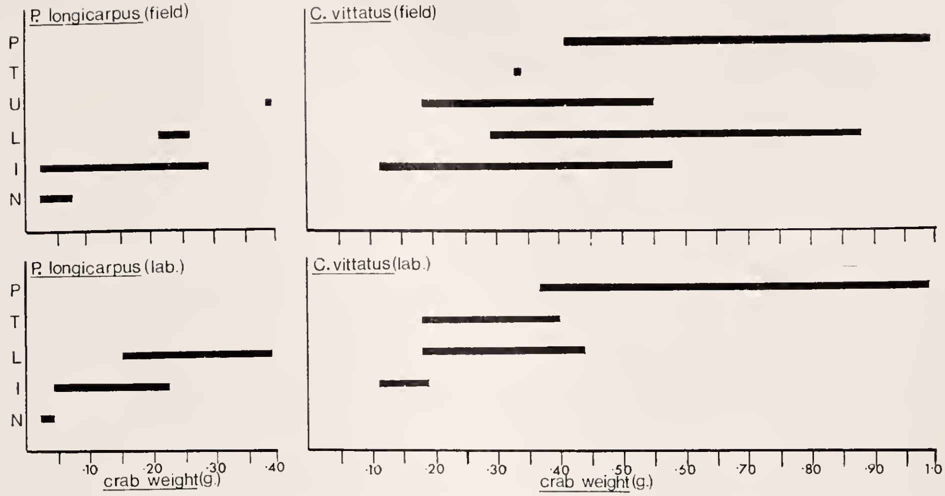

FIGURE 1. Effect of increasing weight of crab on shell species inhabitation. Black bars represent the weight range within which crabs inhabit a species of shell. Abbreviations of shell species are: P, *Polinices;* T, *Thais;* U, *Urosalpinx;* L, *Littorina;* I, *Ilyanassa;* N, *Nassarius.*

selected to test hermit crab species preference was *L. irrorata.* No significant difference was recorded for this species of shell ($P = 0.36$).

There is, therefore, little doubt that resource partitioning of shells takes place both in the restricted selection conditions of the field, and in the unrestricted choice of shells in the laboratory. The partitioning which takes place in the field is not the same as that in the laboratory, however, as there is a significant increase in the number of *P. longicarpus* selecting *L. irrorata* in the laboratory over those occupying *L. irrorata* in the field ($P = 0.036$). Similarly with *C. vittatus,* there is a significant increase in the numbers selecting *P. duplicatus* in the laboratory ($P < 0.001$), but with an accompanying significant decrease in the numbers selecting *I. obsoleta* ($P = 0.05$) and *L. irrorata* ($P = 0.015$). The two-tailed binomial test was again used to calculate all probabilities.

The influence of crab size on shell selection is shown in Figure 1. *P. longicarpus* shows a clear pattern in the laboratory selection of shells. As its size increases, so the species of shell which it prefers changes. Up to 0.04 g it prefers *Nassarius vibex.* From 0.05–0.15 g it prefers *I. obsoleta.* There is then an indeterminate zone from 0.16–0.22 g in which the crab may choose either *I. obsoleta* or *L. irrorata.* Finally, at 0.23 g or greater, it prefers *L. irrorata.*

The weight ranges of *P. longicarpus* inhabiting different species of shell in the field are basically similar to those of the laboratory but with *I. obsoleta* making up for the lack of *L. irrorata* shells. Nevertheless, a Mann Whitney U test shows that the weights of *P. longicarpus* in *L. irrorata* shells are significantly larger than those in *I. obsoleta.*

C. vittatus also shows a clear pattern in the laboratory. At a size range of 0.12–0.17 g, *I. obsoleta* is preferred. At a weight of 0.19 g, three shells can be

TABLE III

Substrate selection of Pagurus longicarpus. *Probabilities are calculated by the binomial test and are two-tailed.*

Category tested	Number on sand	Number on mud	Probability
Individually	5	15	0.042
Grouped	3	17	0.002
Mixed	4	16	0.012

chosen, however. These are *I. obsoleta, L. irrorata* and *T. haemastoma floridana.* From 0.20–0.37 g, only the latter two species are selected, while at 0.38–0.44 g, there is again an indeterminate zone where *L. irrorata, T. haemastoma floridana* or *P. duplicatus* could be selected. At weights of 0.45 g or greater, only *P. duplicatus* is selected. On applying a Mann Whitney U test to the crab weights in different species of shell, significant differences are obtained for all species except *L. irrorata* and *T. haemastoma floridana.* These two species seem to be interchangeable within their preferred range for *C. vittatus* but whereas *L. irrorata* shells were twenty times more common than *T. haemastoma floridana* in the experiment tank, they were only chosen 3.6 times more than them. A binomial test shows that a selection of 5 : 18 is significantly different from the expected ratio of 1 : 20 ($P = 0.002$). Hence there is reason to believe that *C. vittatus* prefers the general shape of *T. haemastoma floridana* to that of *L. irrorata.*

The field results of size of crab against species of shell show a similar though less clearly defined pattern. It is interesting to note that *U. cineria,* although occurring over a wide size range of crab in the field, is not selected at any size range in the laboratory.

Interspecific shell fights

At the end of 24 hours, the ten *P. longicarpus* and ten *C. vittatus* still occupied the same species of shell as when they were first put into the tank. This was the case in both series of experiments. In the first series, the ten *P. longicarpus* were in a species of shell unsuitable for their size compared with the *L. irrorata* shells occupied by *C. vittatus,* whereas in the second series, the opposite was true. It was assumed that, if any shell swaps between species did occur in nature, it would be between individuals in a similar situation to those used in the experimental tank. Since no shell swaps did occur in the tank, it can be assumed with a fair degree of certainty that shell swaps between these two species at Beaufort rarely, if ever, occur in nature.

Substrate selection

The results are presented in Tables III and IV. No significant differences were obtained for either species of crab inhabiting either species of shell against sand or mud when tested by a two-tailed binomial test. The results were therefore pooled for each species of crab. Similarly, no significant difference was found between the results obtained before and those obtained after the sand/mud halves of the tank were reversed. These results were also pooled, so that the table shows

TABLE IV

Substrate selection of Clibanarius vittatus. *Probabilities are calculated by the binomial test and are two-tailed.*

Category tested	Number on sand	Number on mud	Probability
Individually	8	12	0.504
Grouped	11	9	0.814
Mixed	11	9	0.814

only the results of the twenty crabs tested for each category in either species. Significant differences in preference of mud over sand were obtained in *P. longicarpus* regardless of what category was being tested, whereas *C. vittatus* had no preference in any category. To test whether presence or absence of the same or of the other hermit crab species had any effect on substrate choice, a chi-squared test of homogeneity was performed on the ratio of sand : mud for the three categories tested in both species. Neither *P. longicarpus* ($P < 0.80, > 0.70$) nor *C. vittatus* ($P < 0.70, > 0.50$) showed any significant change in preference in the three categories.

DISCUSSION

The results of the shell selection experiment indicate that two major factors operate in determining shell inhabitation by both species of hermit crab in this area. The most obvious factor is that the species of shell which a hermit crab prefers is related to the size (weight) of the crab. In the laboratory there is a clear pattern of shell species inhabitation which is similar for both species of crab. That is, given a crab of a certain weight, it can be predicted which species of shell it will prefer compared with another species. Only in overlap zones (for example, in *C. vittatus* from 0.38–0.44 g) is there some doubt as to the preferred species. It still remains to be discovered, however, whether this apparent species preference is related to the dimensions or specific shape of the shell. This problem is the subject of a second paper on shell occupation of these species (in preparation).

The other major factor operating in determining shell inhabitation is the availability of certain shells. Evidently some species of shells are not available in the numbers that would allow every hermit crab to be in a preferred shell. If this had been the case then there would have been no significant differences between the numbers of shells each species picked up in the field and the numbers of shell species selected in the laboratory. It is worth noting, however, that the ratio of shell species found in the field agrees much closer to that found in the laboratory in the case of *P. longicarpus* than *C. vittatus.* Of the sixty crabs of each species tested, forty *C. vittatus* chose a shell of a different species from the one they were inhabiting in the field compared with only fifteen *P. longicarpus* changing shell species. This indicates that the finite number of shells in this area affects *C. vittatus* more than *P. longicarpus.*

The results of the interspecific shell fights experiment show that *C. vittatus* did not obtain a preferred shell from *P. longicarpus* in the laboratory. Conversely, *P. longicarpus* was unable to obtain a preferred shell from *C. vittatus.* The former result is somewhat surprising in relation to the results of Wright (1973). Wright found that *C. vittatus* was able to obtain the shells of *P. longicarpus* even when *P. longicarpus* was the larger crab. The only instance he cited of *C. vittatus* not being able to do so was when *P. longicarpus* occupied a shell on which living *Hydractinia echinata* were present. No *H. echinata* were present on any of the shells used in the present experiment, however. Provenzano (1959) records a break in the distribution of *P. longicarpus* at Southern Florida, and also records differences in pigment and overall color between Eastern Atlantic specimens and those from the west coast of Florida. From this evidence he divided them into subspecies and it may be that these subspecies have different behavioral as well as morphological properties. Provenzano (1959, p. 404) suspected this when he wrote "experiments in comparative behavior of morphologically similar and dissimilar members of the same genus might yield interesting results" in relation to *P. longicarpus* and *Pagurus pollicaris*. One other possibility is that *P. longicarpus* is in the middle of its geographical distribution at Beaufort, North Carolina, whereas *C. vittatus* is at the northern extremity of its distribution. In Texas where Wright worked, the opposite is the case. *P. longicarpus* is at the southern extremity of its distribution whereas *C. vittatus* has been reported as far south as Brazil (Provenzano, 1959). It may be that a species is more aggressive when it is in an environment which is better suited to its physiological needs.

The shell selection and aggression results help to explain most of the observed shell inhabitation of the two species of hermit crabs but do not entirely explain the small percentage of *P. longicarpus* occupying *L. irrorata* in the field. Approximately one in three of the *P. longicarpus* tested was large enough to occupy an *L. irrorata* shell compared with almost all the *C. vittatus* being large enough. This should therefore have given an *L. irrorata* species distribution of about 1:4 in favor of *C. vittatus*. The actual distribution, 3:37, is significantly above this ratio so that some factor other than chance seems to be responsible. This factor is probably the substrate preference of *P. longicarpus* for mud rather than sand. Since *C. vittatus* does not have this preference, a *C. vittatus* individual is much more likely to come across an empty *L. irrorata* shell than a *P. longicarpus* individual, assuming that *L. irrorata* shells are not washed far from the sand substrate on which they occur naturally.

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SUMMARY

Three series of experiments were conducted on *Pagurus longicarpus* and *Clibanarius vittatus* at Beaufort, North Carolina. The first series showed that the preferred species of shell by either species of crab is determined by the size of the crab and that certain species of shells are not available in preferred numbers for the hermit crab species. This affects *C. vittatus* more than *P. longicarpus*. The

second series showed that shell fighting is not an important factor in determining which species occupies which kind of shell while the third series showed that a substrate preference for mud by *P. longicarpus* may limit it from obtaining a preferred species of shell that occurs naturally on sand.

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A THRESHOLD SIZE FOR METAMORPHOSIS IN THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.)

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There exists convincing evidence that the onset of metamorphosis is accompanied by a decrease in the titer of juvenile hormone (JH) (Wigglesworth, 1970; Gilbert and King, 1973). The *corpora allata* (CA) cease to secrete JH during the final larval instar in most species of insects (Patel and Madhavan, 1969; de Wilde, de Kort and de Loof, 1971; Nijhout and Williams, 1974b) or during the penultimate instar in certain hemimetabolous insects (Ozeki, 1965; Johnson and Hill, 1973). The ensuing decline in the hemolymph titer of JH is the immediate cause of metamorphosis. Many studies have focused on this aspect of the endocrine control of insect metamorphosis.

While the role of JH titer decline in metamorphosis is well established, the mechanism controlling the timing of this event is not well understood. Classical experiments by Wigglesworth (1970) and Fukuda (1944) have demonstrated that the CA do not simply "count the instars" and shut off at a predetermined stage. It is therefore likely that timing of metamorphosis somehow relates to the stage of development that the larva has attained. A clear distinction should be made at the outset between factors which determine whether or not a molt shall proceed and those which determine the character of the molt. It is the latter factors that the present paper is concerned with. In its simplest form the problem can be stated as follows: on the basis of what type of information does a larva decide in which instar its CA are to be turned off? Recent studies by Riddiford (1970a, b) and Riddiford and Truman (1972) have demonstrated the existence of a "program" for postembryonic development in *Pyrrhocoris apterus, Oncopeltus fasciatus* and *Hyalophora cecropia* which determines the instar during which the CA will be inactivated. This program can be disrupted by an application of JH during late embryonic life. Animals treated in this way hatch and grow as normal larvae but fail to metamorphose. Instead, they may undergo a number of supernumerary larval molts and/or transform into larval-adult or larval-pupal intermediates due to the fact that their CA do not turn off during what is normally the final larval instar.

Allegret (1964) has shown that in the wax moth, *Galleria mellonella,* the "program" for the onset of metamorphosis is flexible and can be modified during postembryonic life. By isolating larvae at various stages of development on protein-free beeswax, he found that a larva becomes committed to metamorphose during the presumptive penultimate larval instar. The decision to metamorphose is apparently made at the outset of this instar but can still be modified by the amount of growth that occurs early in the instar. Unless a larva is allowed to grow for at least 24 hours during the presumptive penultimate instar, the CA remain active and normal larval molting continues indefinitely. Experimental alteration of the pattern of growth provides a powerful tool for the analysis of the processes that control molting and metamorphosis (Nijhout and Williams, 1974a, b). In the present paper I will demonstrate that factors associated with the somatic size of larval *Manduca sexta* play a decisive role in controlling the number of larval instars and hence the onset of metamorphosis.

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MATERIALS AND METHODS

Manduca sexta larvae were reared in individual containers as described by Truman (1972) at 25° C under a 12L : 12D photoperiod. Measurements of head capsule size were made by means of a calibrated ocular micrometer mounted in a dissecting microscope. The head capsule size was measured at its greatest width, at the level of the most anterior larval ocelli. Weights of larvae "at the time of molting" refer to individuals taken 12–24 hours prior to ecdysis at the time that their old head capsule had slipped forward and formed a muzzle over the developing head capsule of the new instar.

RESULTS

Parameters of growth of Manduca *larvae*

Under the standard rearing conditions described above, *Manduca* larvae grow at a uniform rate and, without exception, undergo 4 larval molts before pupating. In soft-bodied larvae, such as those of *Manduca,* no striking changes in size occur when the larva molts from one instar to the next, and all somatic growth is confined to the intermolt period. For a given instar the maximum potential size that a larva can attain is most accurately estimated from the dimensions of a sclerotized part of its body. The head capsule is usually used for this purpose as it is one of the few parts that does not change in dimensions during the intermolt. Figure 1 is a plot of the weight of larvae at the time of the molt *vs.* the head capsule width of the succeeding instar. The dimensions of both parameters vary according to Dyar's rule (Dyar, 1890), *i.e.,* there is a constant growth ratio (1.6 for the head capsule width and 5.7 for the weight at the time of the molt) from instar to instar. Hence, the points show a good approximation to a straight line in a double logarithmic plot. This relationship has also been demonstrated in *Ostrinia nubilalis* by Beck (1950).

In order to determine whether a functional relationship exists between the weight that a larva achieves at the time of the molt and size of the new head capsule, I starved 4th instar larvae at various weights so that the molt to the 5th instar occurred at a subnormal weight. Figure 2 is a plot of the weight at which these starved larvae molted and the head capsule size of the resulting 5th instar. It is evident that a continuous range of 5th instar head capsule sizes can be produced by altering the weight at which the molt to this instar occurs.

The results of this experiment suggest that the mass of a larva at the time of the molt is instrumental in determining the size of the new head capsule, and hence, the potential size of the new instar. Figure 2 shows that the relationship

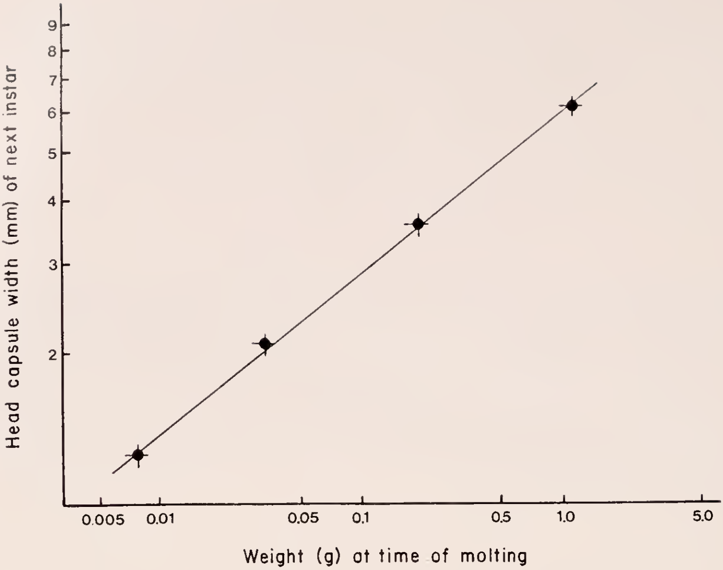

FIGURE 1. Double logarithmic plot of the weight of larvae at the time of molting *vs.* the head capsule width of the new instar for the 2nd through 5th larval instars of *Manduca sexta.* Each point represents the mean of 40 larvae. The bars indicate standard deviations.

between the weight at the time of the molt and the head capsule width of 5th instar larvae, derived from starved 4th instars, deviates significantly from the curve in Figure 1. This discrepancy is of interest and will be investigated in a future communication.

Effect of somatic size on the nature of the subsequent molt

In *Manduca,* and presumably most species of insects, metamorphosis does not occur until a certain minimum, species-specific size is attained. In analogy to the situation in *Galleria* (Allegret, 1964) it should be possible to delay or inhibit metamorphosis by preventing a larva from attaining its full potential size. In view of the fact that 90% of the growth of a larva occurs during the 5th (final) instar, I chose that stage for experimental manipulation. To prevent these larvae from attaining their full potential size, I starved them at various weights and observed the nature of the subsequent molt. Figure 3 illustrates the percent survival of final instar larvae as a function of the weight at starvation. When starvation was begun at a weight less than 3 g, few larvae survived to the next molt. Observations on larvae that died indicated that death was most likely due to dehydration. Indeed, when larvae were allowed to feed on a small block of 2% agar they survived several days longer than those that had no access to water. When these starved 5th instar larvae molted, they did not all pupate. Figure 3 also shows that when larvae weighing less than 4 g were starved, many molted into non-viable larval-pupal intermediates. The pattern of larval and pupal cuticle of these intermediates was identical to that obtained after application of JH

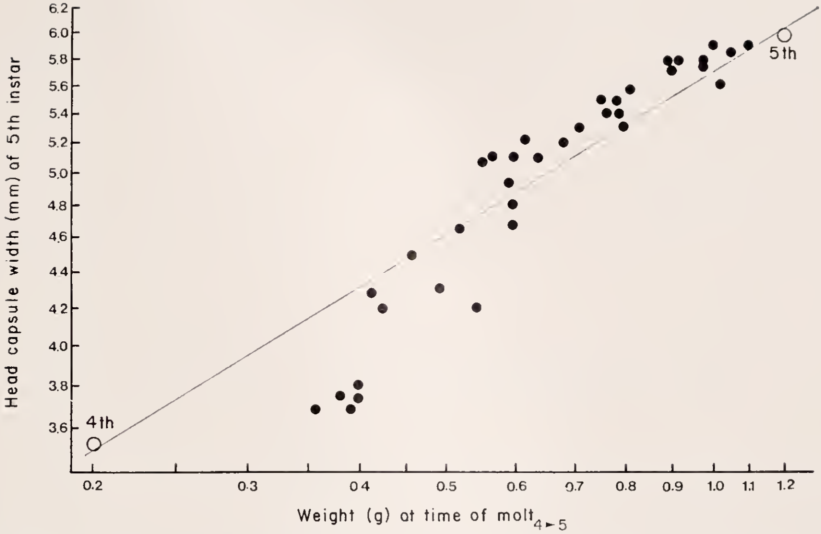

FIGURE 2. Plot of the weight at the time of molting of 4th instar larvae, starved at various weights, *vs.* the head capsule width of the resulting 5th instar larvae. The mean values for normal 4th and 5th instar larvae are indicated by the large open circles. The curve connecting these points is taken from Figure 1.

(Truman, Riddiford and Safranek, 1974). When larvae were starved at weights greater than 4 g, all eventually transformed into small but otherwise normal pupae. It was impossible to obtain viable additional larval instars by simple starvation of a final instar larva.

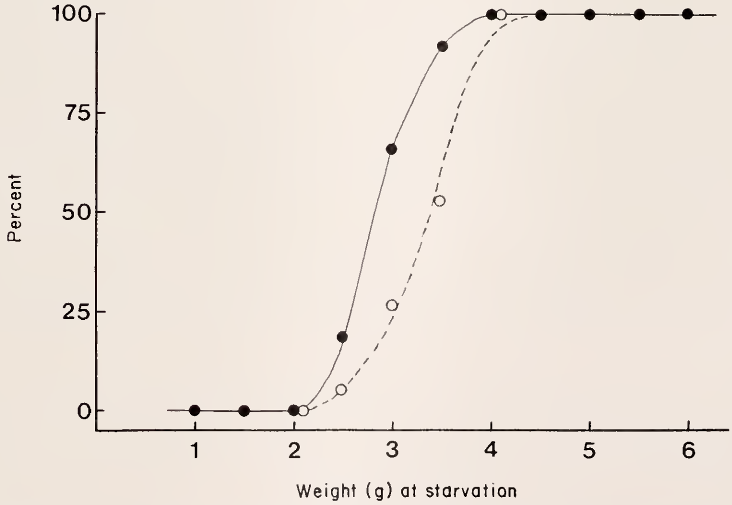

FIGURE 3. Filled circles, percent of 5th instar larvae that survive to molt after starvation various weights. Open circles represent percent surviving 5th instar larvae that molt to normal pupae. The remainder formed inviable larval-pupal intermediates.

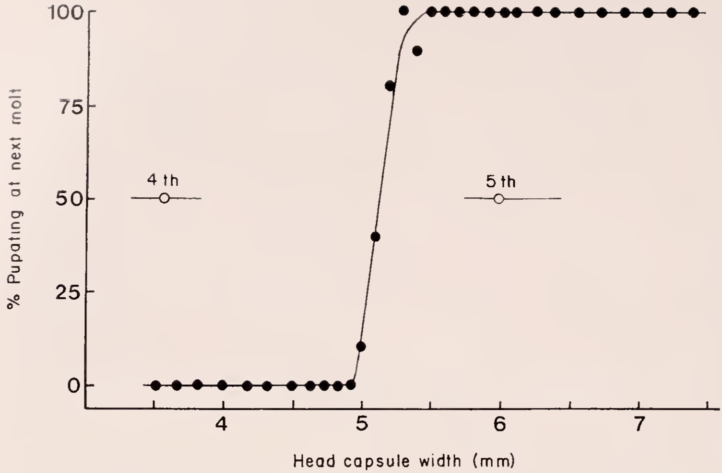

FIGURE 4. Percent of larvae with various head capsule sizes that will pupate at the succeeding molt. The mean (range) head capsule size of normal 4th and 5th instar larvae is indicated. Head capsule sizes were experimentally altered by starving 4th instar larvae at various weights. For detailed explanation, see text. Each point represents 9–25 larvae.

One variable that was not altered by these starvation experiments was the size of the exoskeleton of the larvae since the initial size of all individuals was identical. To determine whether the initial size of a 5th instar *Manduca* larva had any effect on the subsequent fate of the individual, I produced 5th instar larvae of various sizes as described under section 1 of results.

Figure 4 illustrates the fate of 5th instar larvae with various head capsule sizes. A sharp transition in the nature of the subsequent molt occurred at head capsule sizes intermediate between those of normal 4th and 5th instar larvae. The midpoint of this transition was at a head capsule size of 5.1 mm. Larvae with head capsules larger than 5.4 mm formed normal pupae. Those with head capsules measuring less than 5.0 mm always molted into an additional larval instar. The 6th instar larvae produced in this way were quite variable in size but were generally larger than a normal 5th instar. Those 6th instars derived from 5th with head capsules close to the threshold size of 5.1 mm were quite enormous. The head capsule sizes of these 6th instars were also included in Figure 4.

In three cases I obtained 6th instar larvae that were still of subthreshold size. This was due to lethargic feeding and slow growth. These individuals molted into a 7th instar before pupating. The head capsule sizes of these animals are listed in Table I.

In order to determine whether the threshold size depended on the size of the penultimate (4th) larval instar (*cf.* Allegret, 1964), I starved 3rd instar larvae at various weights thus inducing them to form 4th instars of various initial sizes (Figure 5). Fourth instar larvae with head capsules smaller than 3.2 mm (average width 2.95 mm) were selected for further observation. These larvae were allowed to resume feeding and proceeded to molt to 5th instars with a large range of head capsule sizes. Again, the fate of these 5th instar larvae depended only on the

TABLE I

Head capsule sizes (mm) of larvae starved as 4th instars which proceeded to form a 5th, 6th and 7th instar.

Larva	Instar number			
	4	5	6	7
1	—	4.19	5.15	7.10
2	—	4.36	4.80	6.40
3	—	4.38	5.00	6.10
Normal mean	3.55	5.95	—	—

size of their head capsule. Figure 6B shows that the transition in the nature of the subsequent molt occurred once more at a head capsule width of 5.1 mm. If the threshold size had been proportional to the size of the penultimate instar, the transition would have occurred at a head capsule width of about 4.3 mm.

Finally, the degree of heteromorphosis is also affected by the size of the larva (Staal, 1971). Although larvae of *Manduca* exhibit little heteromorphic development, the 5th instar larva is readily recognized by its smooth green integument, lacking the white tubercles that dot the body of the earlier instars. These tubercles are particularly evident on the head capsule of the 4th instar larva. Fifth instar larvae of intermediate sizes are also intermediate on this morphological character, showing progressively fewer and smaller tubercles as head capsule size increases. Figure 6A shows that the transition of morphology from the 4th to the 5th instar occurred at a head capsule width of 4.7 mm.

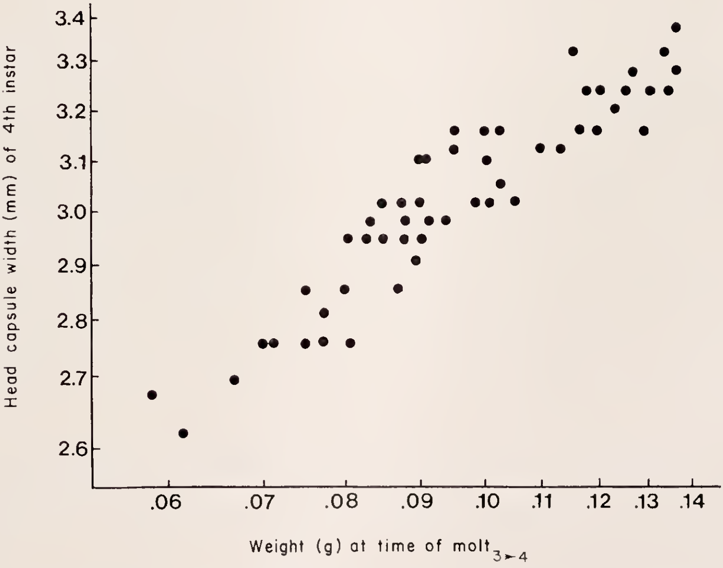

FIGURE 5. Plot of the weight at the time of molting of 3rd instar larvae starved at various weights *vs.* the head capsule width of the resulting 4th instar larva.

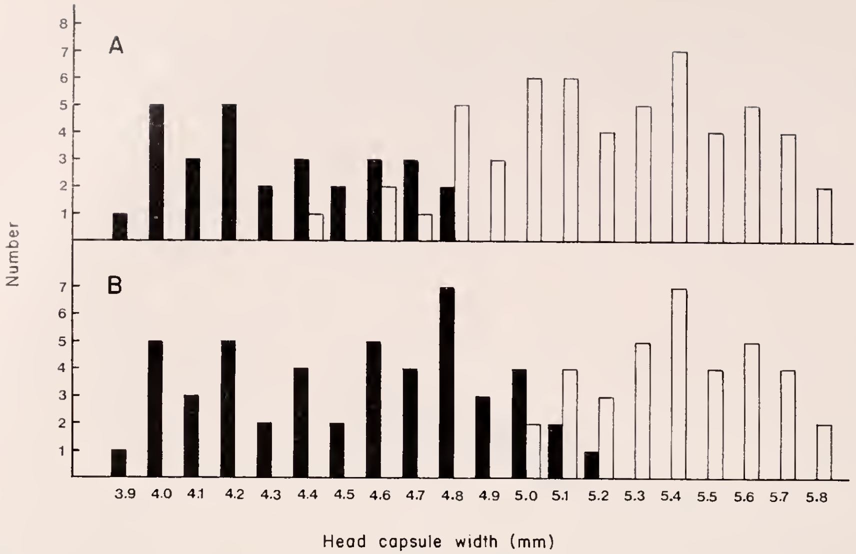

FIGURE 6. (A) Histogram showing the transition in morphology of head capsule and thoracic segments with increasing head capsule size. *White* indicates larvae possessing white tubercles typical of the 4th instar. *Black* indicates larvae with a smooth green integument typical of the 5th instar. (B) Histogram showing the number of 5th instar larvae with various head capsule sizes which pupated (white) or formed a supernumerary larval instar (black) at the succeeding molt. All larvae were derived from temporarily starved 3rd instars (*cf.* Figure 5).

DISCUSSION

According to Bounhiol (1938), larvae of Lepidoptera undergo a "period of indispensable nutrition" before they become competent to molt. In the case of *Manduca* this would be the period needed to grow to a weight of about 3 g during the 5th instar (Figure 3). Nijhout and Williams (1974a) have shown that pupation in *Manduca* occurs progressively later as larvae are starved at lower weights. The limit to the period in which the molt can occur is set by the death of the larva. Therefore, it is likely that the period of indispensable nutrition will depend on the length of time that a starved individual can survive. Since death of starved larvae appears to be largely due to dehydration, the rate of dehydration will determine the length of the period of indispensable nutrition. If the rate of dehydration were faster, the period of indispensable nutrition would be longer and *vice versa*.

When 5th instar larvae were starved at weights below 4 grams, a large percentage of the individuals transformed into larval-pupal intermediates. This was due to the fact that they had not been allowed to attain the critical weight (5 g) at which the CA are inactivated (Nijhout and Williams, 1974a, b). Consequently, the molt occurred in the presence of a substantial titer of JH.

Under the laboratory conditions described in the methods, larvae of *Manduca sexta* invariably have 5 instars. This observation suggests that the number of

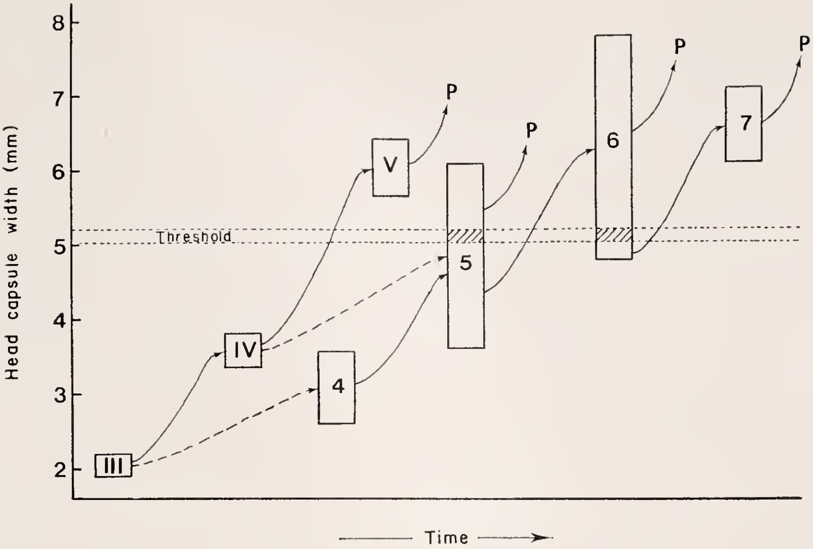

FIGURE 7. Diagrammatic summary of the experiments described under results. The vertical dimensions of the boxes indicate the range of larval head capsule widths found in each group. Roman numerals indicate larvae growing under standard laboratory conditions. Arabic numerals indicate larval instars derived from 3rd and 4th instar larvae that had been temporarily starved (dashed lines). Larvae with head capsules smaller than threshold size (5.1 mm) molt to an additional larval instar. Those with head capsules larger than threshold size pupate (P).

larval instars in this species is a determinate character and that the 5th instar is the final instar.

When 3rd or 4th instar larvae were temporarily starved and allowed to molt at a subnormal weight, the resulting 5th instar larvae were proportionally smaller, as measured by their head capsule sizes (Figures 2 and 5). The subsequent fate of these individuals depended on their absolute size. Only larvae with a head capsule above a threshold size of 5.1 mm pupated at the next molt. Smaller larvae underwent additional larval molts (Figures 4 and 6B). In a few instances the 6th instar larvae that resulted were also below threshold size for metamorphosis and formed a 7th instar (Table I). Larval-pupal intermediates were never formed in these experiments. These experiments are summarized in Figure 7. Except for size, supernumerary larval instars, as well as the pupae, that were obtained were normal and viable.

The data presented in Table I and Figure 6B indicate that the threshold size for metamorphosis does not depend on the size of the preceding instars. Rather, it appears to be a pre-coded threshold of absolute size which is not affected by the developmental history of the individual. The number of larval instars is therefore an indeterminate character. A larva does not "count instars" but rather, it continues to grow and molt until it reaches a certain threshold size. The instar in which this threshold (corresponding to a head capsule width of 5.1 mm) is reached or exceeded is then the final larval instar. The results presented above

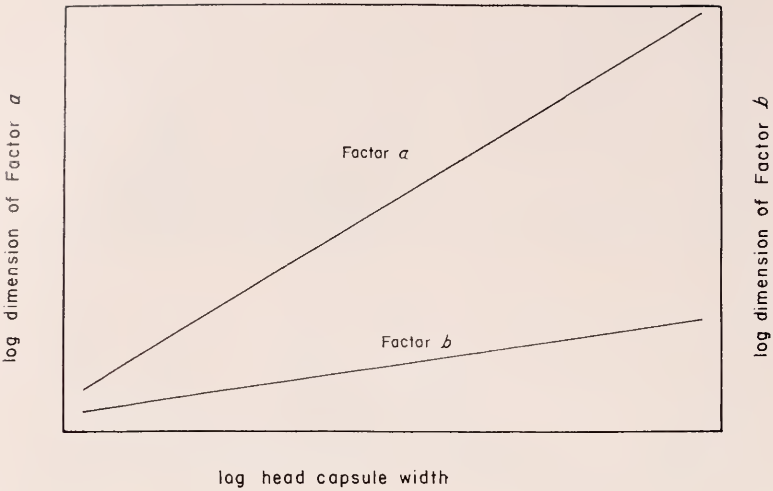

FIGURE 8. Dimension of two factors (*a* and *b*) at the time of molting versus the head capsule size of the next instar. For explanation, see text.

suggest that the commitment to metamorphose is made at the outset of the instar; possibly at the time of the molt when the new head capsule and body sizes are established.

To my knowledge, this is the first time that a threshold of absolute size has been recognized in the control of metamorphosis of an insect. The situation in *Manduca* is therefore quite different from that in *Trogoderma* where Beck (1971a, b; 1972) was able to show that metamorphosis did not depend on factors associated with size or weight. Allegret (1964) showed that larvae of *Galleria* isolated on a protein-free diet proceeded to metamorphose only if isolation occurred when the larvae exceeded a weight of about 18 mg. Larvae isolated at lower weights survived for long periods (60–100 days); during this time they failed to grow and only larval molts took place. Allegret apparently did not attach any significance to the actual dimensions of the larva at the time of isolation. His discussion of the results is restricted to the definition of a critical period during the penultimate larval instar, at which time the larva becomes committed to metamorphose. Unlike *Galleria,* larvae of *Manduca* become committed to metamorphose only at the time of the molt to the final larval instar.

The sharp transition in the nature of the subsequent molt with increasing larval size (Figure 4) suggests that the size-monitoring mechanism used by the larva is highly accurate. The question that now arises is: how can an animal assess its own size so that it may choose a radically different path of development once a pre-set threshold of *absolute* size has been reached? The simplest, if not the only, way in which an individual can monitor its own absolute size is through some form of allometry, *i.e.,* by monitoring the relative growth of one part of its body in relation to some other part or to the body as a whole. Figure 8 illustrates a possible model for such a mechanism. Consider two factors, *a* and *b* (which may be morphological or chemical), that vary allometrically with the size of the larva but have different growth constants. In this case, for purposes of illustration, a stretch receptor mechanism could serve this function. Factor *a* could then be the distance between the attachment points of the receptor spindle, and factor *b* the unstretched length of the spindle. The ratio between these two factors continually changes as the animal grows. As the individual grows the receptor does not "keep up" and becomes progressively more stretched. Therefore, at each instar, the receptor has a higher spontaneous firing rate. It is conceivable that an individual can recognize when the firing rate exceeds a certain threshold value. The point at which this threshold value is reached is the "critical ratio of factors *a* and *b*". Since time plays no role in true allometric growth, the critical ratio of factors *a* and *b* is always reached at a particular absolute size of the individual irrespective of the time (or the number of molts) needed to reach this size. The instar in which the critical ratio of factors *a* and *b* is attained or exceeded will be the final larval instar during which the CA are shut off. At this time it is not possible to say anything about the nature of the factors that the larva uses to assess its size, or the pathway by which these factors influence the CA.

The somatic size of an individual plays a key role in guiding the pattern of development of *Manduca* in two distinct ways. First, Nijhout and Williams (1974a, b) have shown that the release of the prothroacicotropic hormone (PTTH) is inhibited in the presence of JH and that the inactivation of the CA during the final larva instar depends on the attainment of a critical weight of about 5 grams. As soon as the JH has disappeared from the hemolymph the larva proceeds to secrete PTTH and initiates the pupal molt. Consequently, the inactivation of the CA not only determines that the next molt will be a pupation but it also controls the exact timing of this event. Secondly, in the present paper I have presented evidence for the existence of another size-dependent mechanism that determines which instar is to be the final larval instar. By means of this mechanism the larva, in effect, monitors its own absolute size. Only when a sharply defined threshold of absolute size is exceeded does the first mechanism, responsible for the inactivation of the CA, enter into play.

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SUMMARY

1. Under standard laboratory rearing conditions, caterpillars of *Manduca sexta* invariably go through 5 larval instars before pupating.

2. Evidence is presented that the head capsule width of a given instar is proportional to the weight that the individual had attained at the time that the molt to that instar occurred. Fifth-instar larvae with a large range of head capsule sizes

were produced by temporarily starving 3rd and 4th instars, thus inducing them to molt at subnormal weights.

3. Further observations on such larvae revealed that individuals with head capsules wider than 5.1 mm proceeded to pupate at the following molt whereas larvae with smaller head capsules underwent a supernumerary larval molt.

4. It was concluded that larvae of *Manduca* simply continue to grow and molt until they reach or exceed a sharply defined threshold size (corresponding to a head capsule size of 5.1 mm). The instar in which this threshold size is attained is then the final larval instar during which the *corpora allata* will be inactivated.

5. This threshold size for metamorphosis is absolute and does not depend on the prior growth history of the larva. An allometry model is presented for a mechanism by which an animal could "measure" its own absolute size.

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EFFECTS OF HYDROGEN ION CONCENTRATION ON THE MORPHOLOGY OF HEMOCYTES OF THE MOLE-CRAB *EMERITA ASIATICA*

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In previous studies (Ravindranath, 1973, 1974a, b) attempts have been made to extend Jones's classification of hemocytes of insects to the hemocytes of non-insect arthropods, for this scheme is based on morphological criteria. Recently some investigators expressed the view that morphologically different types of hemocytes may represent developmental and functional phases of a single basic cell type (Scharrer, 1972; Price and Ratcliffe, 1974). Gupta and Sutherland (1966) suggested that physiological changes in the hemolymph alter the morphology of hemocytes to a considerable extent, but very little experimental work has been undertaken to verify this suggestion. Such an attempt to analyze hemocyte morphology under different experimental conditions is undoubtedly required before any truly objective definition of hemocyte morphology can be given.

Accidental observations indicate that alteration of the pH of the surrounding medium of the hemocytes may bring about a change in the morphology and behavior of the hemocytes (Grégoire, 1953; McLaughlin and Allen, 1965; Noble, 1970). The present study aims at studying the effects of pH, if any, on the morphology and behavior of hemocytes of *Emerita asiatica*.

Materials and Methods

Specimens of the mole-crab, *Emerita asiatica,* collected from a sandy shore were used within six hours, and observations were made on sexually mature females with carapace length ranging from 30 to 40 mm.

All animals were bled by amputation of the first thoracic appendage, thus minimizing the variations in the blood picture which can result from the use of different bleeding techniques (Jones, 1962). Care was taken to avoid mixing of sand particles or sea water from the appendage with the blood sample.

For studying the effect of pH on the morphology of hemocytes the procedure employed by Grégoire (1953) and McLaughlin and Allen (1965) was followed. A drop of hemolymph issuing from the severed leg was allowed to fall in approximately twice its volume of buffer solution placed into the middle of a glass slide. A thin coverslip, wet in its middle part with the buffer at the pH tested, was gently placed over the hemolymph-buffer mixture. Spontaneous spreading gave a film of suitable thinness for observation. With this procedure, Grégoire (1953) noted that the labile hemocytes are protected from contact with glass surfaces before being thoroughly mixed with the test solution. Control preparations were made with bidistilled water, in order to appreciate the part played by the dilution of the hemolymph and by the pH in the modification of the hemocyte pictures. Unavoidably, the above method of analysis introduced three factors that could possibly influence the results: (1) exposure of the hemolymph to air, (2) the pressure of the coverslip, and (3) osmotic and ionic conditions. The exposure to air was minimized by bringing the cut end of the appendages quickly in contact with the drop of buffer already on the slide. The effect of coverslip pressure on the hemocytes appears to be negligible, for, the changes in the morphology of hemocytes were observed to commence only after about two minutes in all preparations. Besides, uniform trends in the results were obtained in all the six specimens examined with reference to each pH. The buffer systems employed in the present study include Sörensen's M/15 Phosphate buffer, 0.2 M Acetate buffer and 0.02 M Tris-HCl buffer. The major observations were made using Tris-HCl buffer system. One of the advantages of this buffer system is that the buffer solutions of varying pH can be prepared without altering the ionic strength appreciably (Bates, 1961).

The experimental analysis with reference to each buffer series was repeated twice. Each time, three animals were used for every pH of the given buffer. After analyzing the hemolymph with different levels of pH for any particular buffer this way, the analysis was repeated again for the same buffer series. One advantage in experimenting in this way lies in the capacity to carry out detailed observations and thereby detect features which might be overlooked in attempted analysis of larger samples (Arnold, 1969). This method of analysis also facilitates detection of any vague responses. In the present study, the response in question is a shift in the composition of the hemocyte population, which is detectable only by means of differential hemocyte counts.

Differential hemocyte counts were made by classifying a minimum of 200 cells per animal, and the counts were made continuously for ten minutes. The time of addition of hemolymph to buffer solution is taken as zero minute. For studying the effect of pH on aggregation of hemocytes the procedure of Noble (1970) was followed. The hemolymph-buffer mixture was allowed to stand for ten minutes. The number of cell aggregates and the number of cells that were not associated with a cell aggregate, that is, the free cells, were determined. The ratio was expressed as a percentage of total cells.

For interpretation of results, the pH of the hemolymph and pH of the hemolymph-buffer mixture were determined. The hemolymph oozing from the cut end of the appendage was drawn directly into the glass electrode of the pH meter. By this procedure, pH rise due to loss of carbon dioxide is reduced (Stewart, Dingle and Odense, 1966; Bailey, 1971). The influence of dilution of hemolymph with buffers on coagulation of the hemolymph was found out by determining the clotting time, following the procedure described by Peters and Long (1973). All the experiments were carried out between 28° C to 30° C.

Results

Initially, under phase-contrast optics, two categories of hemocytes were distinguished in a fresh drop of hemolymph of *Emerita asiatica*. One type that constitutes about 65% to 75% of the total cells was characterized by the numerous boat-shaped refractile granules. This cell type varied in its length from 18 to 25 μ. The nucleus was spherical and centrally situated. Its presence was sometimes masked by the mass of refractile granules. The other type constituted about

25% to 35% of the total cells, was non-refractile, round, and balloon-like, with a small, round, cart-wheel-like and usually eccentric nucleus. This type fits the description of explosive corpuscles of Hardy (1892) and cystocytes of Jones (1962). Besides these two categories, other types comparable to prohemocytes, plasmatocytes, spherule cells and adipohemocytes of insects (Jones, 1962) were rarely seen (Ravindranath, 1975). All these rare types did not occur in the hemolymph of a single animal.

Effects of pH on the hemolymph

The pH of the hemolymph of intermolt *Emerita asiatica* is 7.95 ± 0.12 at 30° C. This value is in general agreement with pH values of the hemolymph of several decapods (Mangum and Shick, 1973). The pH of the hemolymph-buffer mixture (1 : 2 v/v) varied very slightly with different buffer systems. In Tris-hemolymph mixture, the pH was essentially the same as that of the respective pH value of the buffer system.

In fresh hemolymph, gelification of plasma occurred within 3 minutes at 30° C. The hemolymph-buffer mixture showed variable results. There was no plasma gelification in the acidic range of the pH. Above pH 7.0 gelification is slow and incomplete. The cytoplasmic veils, characteristic of the second pattern of coagulation, described by Grégoire (1970), built up by some disintegrating cystocytes, were infrequently detected by their stretched folds, in the alkaline range of pH. In sodium acetate buffer systems (pH 2.6 to 5.0), the hemolymph precipitated intensely. The precipitation of hemolymph was low or absent in all the pH ranges of phosphate and Tris-HCl buffer systems, respectively.

Effects of pH on aggregation of hemocytes

The results (Fig. 1) show that the percentage of cell aggregates increases with increase in pH of Tris-HCl buffer system.

Effects of pH on the hemocytes

Comparison of hemocytes in preparations diluted with the different pH ranges of buffers, with those of fresh, undiluted or distilled water diluted preparations, gave clear indications of alteration of granular hemocytes in relation to variations in pH. The cystocytes swell and disintegrate in the solution within 3 min. In acetate buffers at pH 3.6, 4.0, 4.4, 4.8, 5.2, and 5.6, and in the phosphate buffers at a range between 6.0 and 6.8, the granular hemocytes underwent a very rapid alteration to spindle-shaped and pseudopodial forms. The refractile boat-shaped granules became fine. The homogeneous appearance of the nucleus changed into heterogeneous.

In phosphate buffer at pH 7.2, 7.6, and 7.8 as well as in all ranges of Tris-HCl buffer, the hemocytes underwent similar changes, but the speed with which the alterations occurred differed with different hydrogen ion concentrations and buffer systems.

Stages of transformation

The alterations observed in the granular hemocytes are classified as follows: *Stage I* (Figs. 2 and 3)—this stage is represented by the unaltered amoeboid

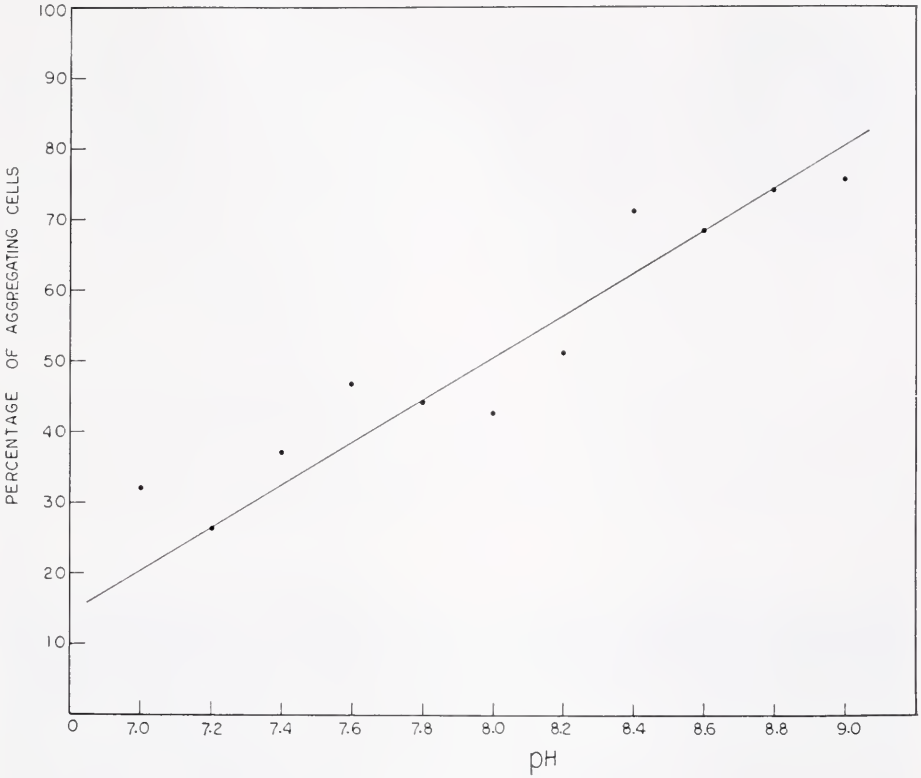


FIGURE 1. The percentage of aggregating hemocytes in different hydrogen-ion concentrations of 0.02 M Tris-HCl buffer system. The ratio of the number of hemocyte aggregates and the number of free cells are expressed as percentage of total cells.

granular hemocytes. *Stage II* (Figs. 2 and 4)—in this stage the boat-shaped refractile granules became fine, and some lose their refractility. The nucleus was visible and appeared to be homogeneous. The cells continued to be amoeboid. *Stage III* (Figs. 2 and 5)—in this stage the granules disappeared completely. The nucleus was clearly visible, and the cells exhibited amoeboid movements in buffer-hemolymph mixtures. Cells of this stage were identical to plasmatocytes described by previous workers (see Jones, 1962). *Stage IV* (Figs. 2 and 6)—in this stage the cell became round and appeared balloon-like, due to vacuolization of the cytoplasm. The nucleus shrunk in its size and became pycnotic, with a reticular chromatin structure, indicative of chromatin disaggregation. The cells were amoeboid. All these features of the cell strongly recall those of the cystocytes. In some preparations, between the buffer range of 8.6 to 9.0, it was noted that the cart-wheel-like nucleus again turned homogeneous and showed distinct signs of swelling of the nucleus. In some cases, cytoplasm was observed to shrink and the cell gave the appearance of a prohemocyte. In the present study such cells were also included in Stage IV. Overlapping of the stages was observed near neutral pH but it does not occur frequently.

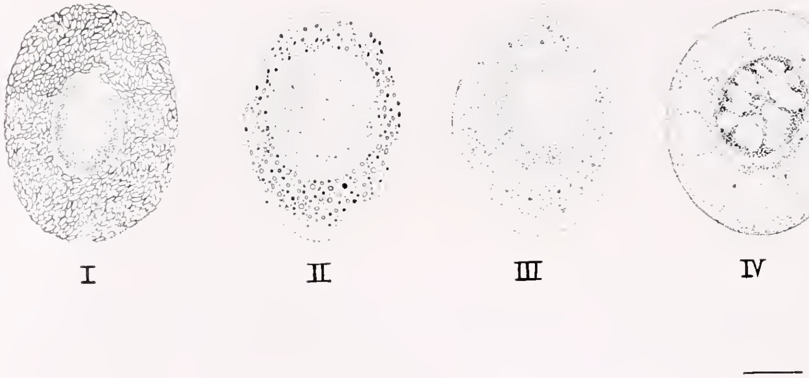
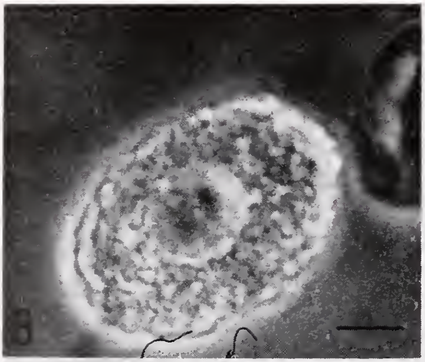
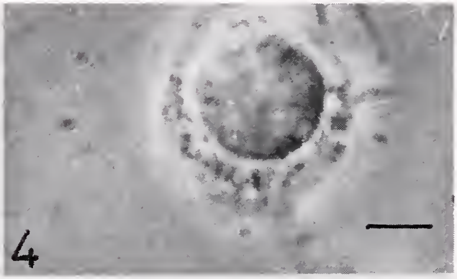
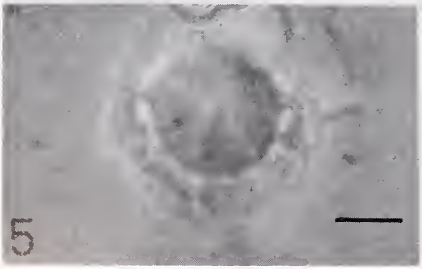


FIGURE 2. The sequential changes observed in a granular hemocyte in the pH range between 7.0 and 9.0 of Tris-HCl buffer. The changes are arbitrarily classified into four stages: I) normal granular hemocyte in unaltered condition in the buffer; II) the second stage of the hemocyte transformation, note the dissolution of the elliptical granules; III) the third stage recalls a plasmatocyte, note the degranulation of cytoplasm; and IV) the final stage recalls a cystocyte, note the vacuolization of the cytoplasm, stiffening of the cell surface and nuclear pycnosis.







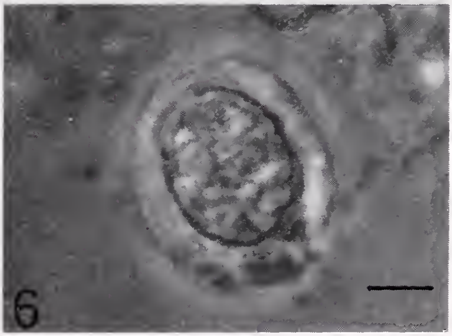


FIGURE 3. Normal granular hemocyte in 0.02 M Tris-HCl buffer at pH 8.2. Note the refractile and elliptical granules and homogeneous nucleus.

FIGURE 4. The second stage of granular hemocyte transformation in Tris-HCl buffer at pH 8.2. Note the dissolution and the loss of refractility of the elliptical granules.

FIGURE 5. The third stage. Note the degranulation of the cytoplasm.

FIGURE 6. The last stage. Note the vacuolization of the cytoplasm, stiffening of the cell surface and the nuclear pycnosis.

Differential counts of the stages of hemocytes

Initially, the differential counts of the above referring to stages of the granular hemocytes were made with reference to phosphate buffer (pH 7.0, 7.6, 7.8, and 8.0). Precipitation of hemolymph in this buffer and the resultant clumping of hemocytes with the precipitate did not permit a detailed analysis. Therefore, the differential counts were made in detail with Tris-HCl buffer system in which precipitation was absent. Moreover, as the ionic strength of Tris-HCl buffer solutions of varying pH is not altered appreciably, the ionic effects of the buffer on the hemocytes may not be significant. The results (Fig. 7) show that the alterations in the morphology of granular hemocytes varied significantly with different hydrogen ion concentrations and times. As the pH of the solution increased from 7.0 to 9.0, the transformation of granular hemocytes slowed down. Correlated with the increase in transformation at pH 7.6, a marked increase in the cystocyte-like cells was observed (Fig. 7). The overall pattern of fluctuations of cystocyte-like cells (Stage IV cells) showed an inverse trend to those of granular hemocytes and cells of Stages II and III. When granular hemocytes and cells of stages II and III showed a maximum retardation in their alteration at pH 8.2, the percentage of Stage IV cells decreased significantly. At all levels of pH, cells of all stages except Stage IV, showed a decrease in their number after ten minutes (Fig. 7).

DISCUSSION

The most striking feature in the composition of the hemocytes of *Emerita asiatica* is the predominance of one cell type, namely the granular hemocyte. A characteristic feature of this hemocyte is the nature of granular inclusions, which are refractile and boat-shaped. This type of hemocyte has been reported in several arthropods (see Ravindranath, 1975). But such a cell type does not fall in any of the categories of hemocytes classified by Jones (1962). His description and the figures of the granular hemocytes (Jones, 1962, 1965) and the granular hemocytes reported in a millipede, *Thyropygus poseidon* (Ravindranath, 1973), correspond to Stage II cells of the present study.

Arnold (1959, 1972), on the other hand, has observed the refractile boat-shaped granules both in plasmatocytes (Fig. 12, 1959; Fig. 104, 1972) and in granular hemocytes (Figs. 105 and 123, 1972) of *Blaberus discoidalis* and *B. giganteus.* Such a striking similarity in the granulation of the cytoplasm of these two different categories of cells emphasizes the need for an objective, unambiguous distinction of plasmatocytes and granular hemocytes (see also discussion in Ravindranath, 1974a). It appears therefore that the classification of hemocytes of arthropods is still in an incomplete stage.

The observations made in the present study with reference to the influence of pH indicate that the pH of the surrounding medium influences both the behavior and the morphology of hemocytes. Comparison of the results with earlier observations is hampered by the fact that previous analyses are predominantly qualitative. However, the observations made here not only confirm the findings of others (Cameron, 1952; Loeb and Blanchard, 1922; Grégoire, 1953; McLaughlin and Allen, 1965; Noble, 1970), but also favor the suggestion of McLaughlin and

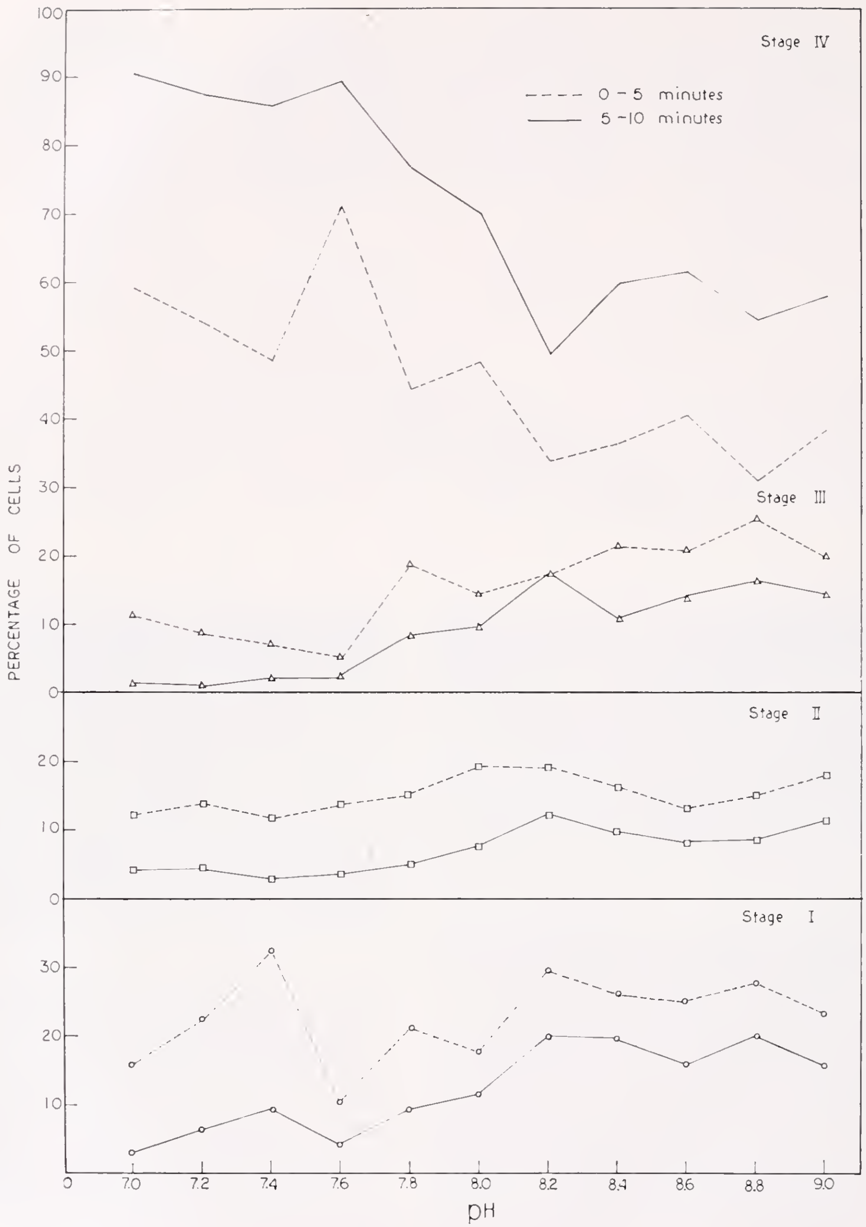


Figure 7. The response of granular hemocytes to variation in hydrogen-ion concentrations of Tris-HCl buffer. The response is detected by means of differential counts of the stages, which is expressed as percentage of total cells.

Allen (1965) that attention to the pH of diluents is important in a study of hemocyte morphology.

Some important features could be made with reference to the pH-induced morphological variations of granular hemocytes, though the experimental conditions may not reflect a true physiological state of the animal. First of all, the pH-induced mutability of granular hemocytes is not at random but sequential; secondly, the stages of alterations of hemocytes are facsimiles of the major arthropod hemocyte types, namely, granular hemocytes (described by Jones, 1962, 1965), the plasmatocytes and cystocytes. The above features suggest that the specific physiological conditions of the animals which bring about a change in hemolymph pH, such as autotomy and molting (Bliss, 1960) may likewise trigger the transformation of one form of hemocyte into another. Although such a suggestion is in general agreement with the views of Gupta and Sutherland (1966), the fact that there are inherent dangers in extending the observations based on *in vitro* experiments to *in vivo* behavior, necessitates further experimental data to confirm the above suggestion.

A feature of significant interest is the formation of cystocyte-like cells in the final stages of transformation of granular hemocytes. This transformation is brought about by degranulation and vacuolization of the cytoplasm and nuclear pycnosis, which may be a result of cell injury or toxicity, or autolysis (Dernby, 1918) caused due to alteration in the pH of the surrounding medium. The effect is minimized at pH 8.2. The above mentioned effects of cell injury or autolysis are also known to be caused as a result of aging of cells (Myers and DeWolfe-Glade, 1964). It is reasonable to infer that cystocytes which also occur in the normal hemolymph of *Hippa* (*Emerita asiatica*) and of other arthropods may be the "end-cell stage" of aging granular hemocytes (see also discussion in Ravindranath, 1975).

The effects of pH on the morphology of cells in vertebrates is well known. The works of Meyer and Sievers (1936), Teorell (1951) and Bittar (1964) indicate that the variations in pH may affect the stability and permeability of hemocyte membranes by altering the strength of the charges of the solution of protein, which exist in and out of the cells. Probably the increase in aggregation of hemocytes with increase in pH could be due to similar alterations in the properties of the cell surface.

An important work that is relevant to the observations made in the present study is that of Dernby (1918), who first applied Sörensen's concept of pH and made an extensive study of autolysis of several tissues as a function of pH, recognizing the existence of an acid optimum and also a second one in the alkaline range. It is evident from the above work, that the observed alterations in the morphology of hemocytes may be indicative of autolysis. The overall pattern of fluctuations of cystocyte-like cells (Fig. 7) is noteworthy in this connection for it indicates increased rate of transformation (autolysis?) of granular hemocytes at two points, one in the acid and the other in the alkaline region.

However it may be, the above results necessitate the importance of determining the pH of the hemolymph wherever different hemocyte counts are made, for the variations observed during different physiological conditions of an arthropod (see Ravindranath, 1974c) could be due to the variations in the pH of the hemolymph that occur during such periods rather than due to the different physiological requirements *per se*.

In conclusion, it is realized that classification of hemocytes into arbitrary types is necessary for purposes of quantitative morphological or physiological investigations, even if the various hemocytes should be found to represent only phases of development of single types (Jones, 1962). Moreover, the different developmental phases of a single cell type could be capable of distinct functions just as truly different cell types are (Price and Ratcliffe, 1974). While classifying the cells it should be noted that a classification based on prepared hemolymph films alone, which has been the common practice, or from any single technique, seems unwise and would definitely mislead in the interpretation of the hemocyte complex of arthropods (see also discussion in Arnold, 1959).

I am grateful to Professor Dr. K. Ramalingam and Drs. M. H. Rajeswari Ravindranath and T. Subramoniam for many valuable suggestions. Sincere appreciation to Mr. V. T. Sridharan, Department of Botany for his valuable help in pH analyses and to Dr. C. V. Subramaniam, Director, Centre of Advanced Study for Botany, for providing necessary facilities.

SUMMARY

Variations in the pH of the surrounding medium of hemocytes *in vitro* influence the morphology of granular hemocytes, the most predominant cell type in the hemocyte population of *Emerita asiatica*. Qualitative studies reveal that in the acidic range these cells become spindle-shaped and pseudopodial. Between pH 7.0 and 9.0 of the Tris-HCl buffer system, the hemocytes show a sequential change. The morphological features of the stages are facsimiles of the major hemocyte types occurring in the hemolymph of *Emerita asiatica*. The granular hemocytes become plasmatocyte- and cystocyte-like cells.

Differential counts of hemocytes and pH-induced variations of granular hemocytes reveal that the rate of change decreases with decreasing pH of the surrounding medium. The aggregation of hemocytes increases with increase in pH.

The pH-dependent variations of hemocytes indicate that for a truly objective definition of hemocyte morphology and for a strict classification into distinctly separate cell types, experimental analyses of hemocyte morphology are required.

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BEHAVIOR AND ELECTRICAL ACTIVITY IN THE HYDROZOAN *PROBOSCIDACTYLA FLAVICIRRATA* (BRANDT). II. THE MEDUSA

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Despite a growing interest in the behavioral electrophysiology of such hydromedusae as those of *Sarsia* (Passano, 1965; Mackie, Passano and Pavans de Ceccatty, 1967; Mackie and Passano, 1968), *Euphysa* (Mackie and Passano, 1968), *Cladonema, Tiaropsis, Staurophora* (Passano, 1965), and *Spirocodon* (Ohtsu and Yoshida, 1973), a comparison of behavior and associated electrical activity in both the polypoid and medusoid phase of one species has not been reported. This study of the limnomedusan *Proboscidactyla flavicirrata* enables such a comparison to be made.

There is a degree of homology and analogy within pulse types and their conducting systems that is surprisingly exact. This applies particularly to epithelial and epitheliomuscular systems (Spencer, 1974).

The medusa of *P. flavicirrata* has proved an admirable experimental animal for furthering our understanding of all the conducting systems and their pulses that have so far been recognized in the hydromedusae.

MATERIALS AND METHODS

The medusa of *P. flavicirrata* (Fig. 1) is locally abundant in coastal waters of the Pacific Northwest between the end of May and late September. Developmental stages between the post-release four-tentacled jellyfish and the sixteen-tentacled stage have not been found. However, such stages are readily obtained from laboratory culture.

Jellyfish were collected from two localities. Those collected from the dock at the Friday Harbor Laboratories (San Juan Island) were taken at night from April to July, whereas those collected at Oak Bay (Vancouver Island) from August to October were collected during daylight. Medusae were kept in battery jars placed on a water-table at 9–13° C. The water, in the jars, was replaced once a day a few hours after the jellyfish had been fed.

Electrical activity was recorded using polyethylene suction electrodes with tip diameters between 20 and 100 μm with Pt or Ag/AgCl wire as the conductor.

Seawater in the recording dish was cooled (11–14° C) by passing water through a glass coil partially sunk into the wax-base of the recording dish. Jellyfish were never pinned down during recording since the electrodes themselves secured the specimens.

Although most of the behavioral observations were made in the laboratory, S.C.U.B.A. equipment was used to observe adult medusae.

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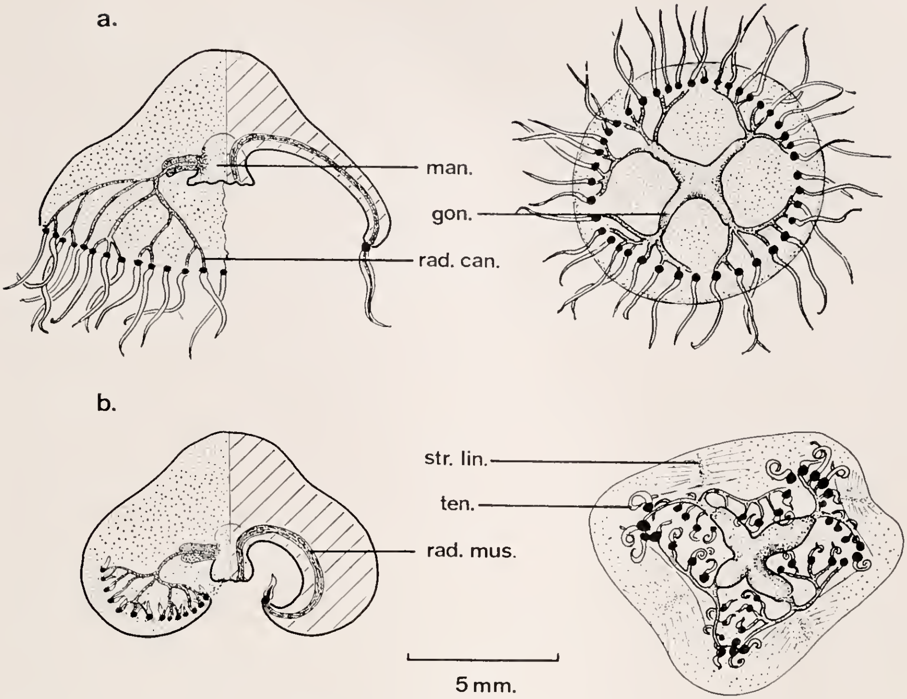

FIGURE 1. Medusa of *Proboscidactyla flavicirrata* at rest (a) and "crumpled" (b) as viewed from the side and above. The side views are diagrammatic and show one hemisphere as a sagittal section. The radial muscle of one perradial canal and the contiguous longitudinal muscle of a tentacle can be seen. The views from above were drawn from photographs: gon.—gonad; man.—manubrium; rad. can.—radial canal; rad. mus.—radial muscle (contracted); str. lin.—stress lines of the exumbrella epithelium; ten.—tentacle (contracted).

RESULTS

Behavioral observations

Feeding. If jellyfish are placed in an aquarium and then several beakers of fresh plankton poured in, jellyfish will start feeding almost immediately. Single tentacles or groups of neighboring tentacles contract while the margin of the bell associated with these tentacles swings in towards the manubrium. As the margin rolls up into the subumbrella cavity the manubrium bends out towards incoming tentacles. If the prey is small the manubrial lips engulf the tentacles, or if large, the tentacles pass the prey directly to the mouth. The food captured in this way consists of small cyclopoid copepods, cladocerans, trochophore larvae, crab zoeae, copepod nauplii and various eggs.

Medusae of *P. flavicirrata* can often be seen motionless in the sea with the tentacles extended horizontally. In this posture they sink slowly. This behavior has been noted in other limnomedusae and is frequently described as "sink-fishing". The others include *Eperetmus typus* (Mackie and Mackie, 1963); *Gonionemus*

(Yerkes and Ayer, 1903); *Limnocnida* (Edney, 1939); and *Craspedacusta* (Russell, 1953). It seems likely that this is a typical feeding pattern of the limnomedusae since *P. flavicirrata* spends much of the time "sink-fishing", interrupted by intermittent radial feeding responses and bouts of upward swimming.

Swimming. Swimming involves symmetrical contraction of the circular, striated muscle sheet that is present throughout the subumbrella ectoderm from the apex of the velum to the base of the manubrium. Each swimming beat causes elongation of the bell and a reduction in the volume of the subumbrella cavity, thus forcing water out through the velar opening. The velum forms a wide-mouthed funnel which tends to increase exhaust velocities by narrowing the opening.

Undisturbed jellyfish swim infrequently. In the sea, jellyfish are only seen swimming if agitated by water currents or after a period of sink-fishing. Jellyfish were rarely seen more than 10 m below the surface during the day and 1 m at night. Mature jellyfish have slight negative buoyancy and must swim in order to remain near the surface, a swimming period lasting 30 sec will displace a large jellyfish vertically about 80 cm. In comparison, young jellyfish (4–16 tentacles) sink more rapidly and presumably have to swim for more extended periods to maintain their depth. Indeed in the laboratory young jellyfish tend to swim more frequently than mature jellyfish.

The swimming contraction is not graded and thus the distance through which a jellyfish swims is determined by the number of contractions in a burst of swimming and not by the extent of each contraction. In young jellyfish the interval between each swimming contraction in a burst is from 250–350 msec and in mature jellyfish it varies from 300–400 msec. Velar-steering was never seen, even if the jellyfish were tilted through 90°. The medusa of *P. flavicirrata* is very stable with the bell uppermost, since a large volume of mesogloea is well separated from the more dense manubrium and tentacles, thus giving a large turning moment.

Synchronous tentacle shortening, involving all the tentacles of a medusa, usually occurs either immediately (less than 1 sec) prior to or during a swimming burst. While the jellyfish rests between swimming bursts the tentacles are held a few degrees below the horizontal.

Crumpling. "Crumpling" is a term first used by Hyman (1940) to describe a defensive response seen in a number of hydromedusan species, both antho- and leptomedusae, involving contraction of radial muscles together with the longitudinal muscles of the tentacles so that the margin and tentacles are drawn up into the subumbrella cavity. Unlike other hydromedusae, the radial muscles of *P. flavicirrata* are endodermal and lie on the subumbrella side of the radial canals where they are restricted to bands either side of the canals (Spencer, 1971). If the radial muscles of all four quadrants contract, then the jellyfish takes on a square outline when seen from above (Fig. 1b). Any portion of the ectoderm of the jellyfish can be stimulated mechanically to elicit a crumple, with the exumbrella and margins being the more sensitive regions, though tentacles must be shaken before a jellyfish will crumple. During crumpling all other muscular activity ceases.

Synchronous tentacle shortening. Synchronous tentacle shortening has already been described in connection with swimming, but it can also be seen in all medusa stages during rest periods. These contractions usually involve every tentacle and

TABLE I

Parameters of the pulse-types.

Pulse type	Initial polarity	Duration (msec)	Conduction velocity (cm/sec)	Amplitude (mV)	
				+	−
Swimming pulses, N = 450	−	30–110 $\bar{x} = 60$	22–26 (14° C)	0.04–0.36 $\bar{x} = 0.16$	0.25–0.80 $\bar{x} = 0.30$
Marginal pulses, N = 120	+	40–90 $\bar{x} = 71$	3.9–5.6 (14° C) (only applies to attached medusae)	0.01–3.20 $\bar{x} = 1.3$	0.02–1.20 $\bar{x} = 0.40$
Tentacle contraction pulses, N = 80	+	40–120 $\bar{x} = 100$	—	0.05–5.00 $\bar{x} = 2.50$	0.03–0.60 $\bar{x} = 0.20$
Crumpling pulses, N = 130	−	10–50 $\bar{x} = 17$	5–22 (14° C)	0.09–1.00 $\bar{x} = 1.80$	0.10–4.00 $\bar{x} = 2.50$

Pre-swim pulses are not included in this table because of lack of sufficient data.

can be single or appear in bursts. Relaxation is immediate, unlike the tentacle contractions during crumpling when relaxation is far slower. Sometimes these contractions are only seen over a short length of the tentacle near the tip, particularly in adult medusae. Often tentacles curl aborally as they contract. Synchronous tentacle shortening is not dependent on external stimulation of any kind.

Electrical activity

In the following account of electrical activity in the medusa of *Proboscidactyla flavicirrata* the term "mature" refers to a released medusa with a complement of at least 40 tentacles and bell diameter exceeding 4 mm, and does not necessarily imply sexual maturity. A summary of the major parameters of the pulse types is given in Table I.

Feeding potentials. Potentials associated with feeding have only been recorded from two released medusae, and thus the following must be treated with caution. These low amplitude, positive going potentials can be recorded from the bases of perradial tentacles actively involved in feeding. They do not appear to be conducted through to neighboring tentacles.

Swimming pulses (SP's) and pre-swim pulses (PSP's). Swimming pulses can be recorded from any part of the circular muscle sheet of the subumbrella and velum in both attached and free medusae. There is a one for one relationship between swimming pulses and swimming contractions. The pulses are always biphasic, with a characteristic shape, which may be preceded by a negative-going spike (Fig. 2). This spike (the pre-swim pulse) is only seen if suction electrodes are attached close to the margin of the subumbrella.

Conduction velocities of SP's have been calculated for mature medusae between electrodes placed on the subumbrella surface near the junction of the velum with the subumbrella. The stimulating electrode is placed between tentacles over the endodermal "ring-canal" at 45° from one of the recording electrodes, and the

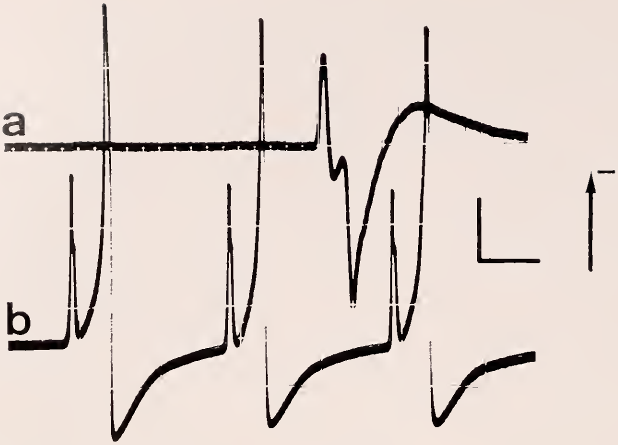

Figure 2. A swimming burst consisting of three contractions of the circular muscle. One electrode (a) was attached to a tentacle and recorded a single tentacle contraction; the other electrode (b) was attached to the margin on the subumbrella surface and recorded three swimming pulses (SP's), each preceded by a pre-swim pulse (PSP). The horizontal bar represents 200 msec and the vertical bar, 100 μV.

recording electrodes are positioned 90° apart. For this preparation a mean value of 25 cm/sec (14° C, $N = 27$) was calculated if it is assumed that the conduction route is circular between recording electrodes. If three recording electrodes are positioned 120° apart at the same vertical height on the subumbrella then the synchrony of excitation of the circular muscle sheet can be observed, with excitation reaching all three sites within 20 msec, normally it is much less than this (4–6 msec at 14° C).

When a mature jellyfish is placed in a solution containing one part of isotonic magnesium chloride and one part seawater spontaneous swimming stops after some

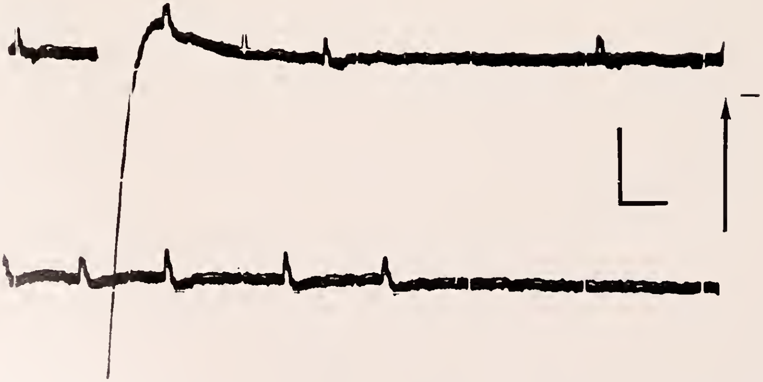

Figure 3. Reappearance of pre-swim pulses (PSP's) after Mg^{++} anaesthesia. The two traces form a continuous recording. The large potential on the upper trace is a marginal pulse. The horizontal bar represents 100 msec and the vertical bar, 50 μV.

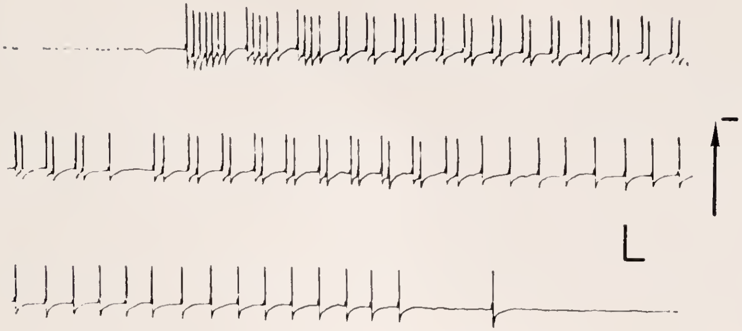

FIGURE 4. Swimming period of a mature medusa. Swimming pulses (SP's) are recorded by an electrode attached to the subumbrella surface. The horizontal bar represents 1 sec and the vertical bar, 500 μV.

15 min. If the water containing excess Mg^{++} is flushed out with fresh seawater, potentials can be recorded from the margin that have a frequency reminiscent of SP's. This happens about 4 min after flushing (Fig. 3). These potentials have a very low amplitude (9–60 μV) and are grouped as bursts of from 4–9 pulses which become bursts of 3, 2, and 1 pulse after 7 min. There is no visible muscular contraction associated with these potentials. Then about 15 sec after bursts with three potentials first appear, swimming contractions can be seen together with the associated SP's. At first these SP's are of low amplitude but some 9 min after flushing with fresh seawater they resemble normal SP's. These swimming potentials seen during recovery from Mg^{++} anaesthesia are probably identical to the pre-swim pulses seen preceding SP's that have already been described.

Swimming pulses can be recorded over long periods of time from both attached and free medusae giving data on temporal patterns of swimming. These swimming patterns are surprisingly regular and typically consist of bursts of swimming pulses with distinct interburst intervals (700 msec—several min) and interpulse intervals (200–500 msec) which in a series form a swimming period (3 sec to several hours), as shown in Figure 4. Table II summarizes the temporal patterns of swimming pulses that were recorded from 4 mature medusae (approximately

TABLE II

Temporal patterns of swimming in medusae.

		Burst type					
		7	5	4	3	2	1
Frequency of burst type	Mature Attached	1% —	2% 0.6%	5% 2%	19% 20%	49% 64%	24% 13%
Mean interpulse interval	Mature	308 msec SD 13	365 msec SD 30	390 msec SD 26	425 msec SD 35	384 msec SD 49	n.a. n.a.
	Attached	— —	225 msec SD 15	266 msec SD 35	301 msec SD 64	360 msec SD 48	n.a. n.a.
Mean interburst interval following stated burst type	Mature	1060 msec —	1003 msec SD 230	902 msec SD 190	982 msec SD 160	1120 msec SD 83	1350 msec SD 90
	Attached	— —	1500 msec SD 290	1135 msec SD 150	975 msec SD 140	1180 msec SD 190	1230 msec SD 240

6 mm diameter) and 6 attached medusae (approximately 600 μm diameter) over a total recording time of 15 hr with swimming periods totalling 2 hr 40 min.

The main points demonstrated in Table II are: (a) the frequency of burst types is different for mature and attached medusae; (b) the mean interpulse interval is longer for mature than attached medusae; (c) a burst type with two pulses is favored in both attached and mature medusae; (d) there is a tendency for the mean interpulse interval to increase as the burst size decreases; and (e) the interburst interval varies only slightly with burst size.

When mature jellyfish are stimulated to swim with just supra-threshold shocks delivered to the margin, the natural frequency at which the muscles are contracting can be overridden. However there is a maximum frequency at which the swimming pulse system can be driven. This occurs when the interpulse interval is some 260 msec, which is also the minimum interpulse interval seen during spontaneous swimming.

Marginal pulses (*MP's*). Marginal pulses can be recorded from the tentacles and marginal regions of both attached and free medusae. Such pulses are often not directly associated with any motor activity, and thus may have an integrative or pacemaker function. Nevertheless, synchronous contractions of all the tentacles are always accompanied by a marginal pulse(s). MP's recorded from attached medusae are seldom grouped in bursts and the system is rarely silent, whereas MP frequency in free medusae is often burst-like and the interpulse interval is rarely as constant as it is in attached medusae. In attached medusae MP firing is synchronized between the four tentacle bases and only occasionally is MP excitation not conducted through to all four tentacles.

Four days after release, medusae begin to develop 4 interradial tentacles. If suction electrodes are attached to the bases of these tentacles, marginal pulses can be recorded, but there is no synchrony of MP firing between these tentacles and any other tentacles at this stage. Marginal pulse firing within this group of four tentacles is also unsynchronized, however, recording taken from the four primary tentacles (perradial) show synchronous firing with each other, and by the 6th day after release MP firing of all eight tentacles is synchronized (Fig. 5). In individuals with the full complement of tentacles MP firing is not necessarily synchronized between all the pacemaker sites since small groups of adjacent tentacles can show unilateral, synchronous firing.

Conduction velocities of MP's have been calculated by electrical stimulation of tentacle tips and from recordings of spontaneous activity in pre-release medusae. A mean value of 4.7 cm/sec (14° C, $N = 15$) is obtained if it is assumed that marginal pulses are conducted in the marginal nerve-ring(s). These velocities only apply to attached medusae since it is difficult to stimulate MP's in mature medusae without exicting the crumpling pulse system.

In attached medusae, using three electrodes, it can be seen that each tentacle base contains a site capable of initiating marginal pulse excitation. One site may lead for many minutes to be rapidly followed by another, yet other sites may show attenuation of MP amplitude and stop firing for a period. Such relationships are far more difficult to see in free medusae since there are numerous tentacles each undergoing independent tentacle contraction (to be described later), and also the bursting pattern of marginal pulses leads to confusion over which pulses are

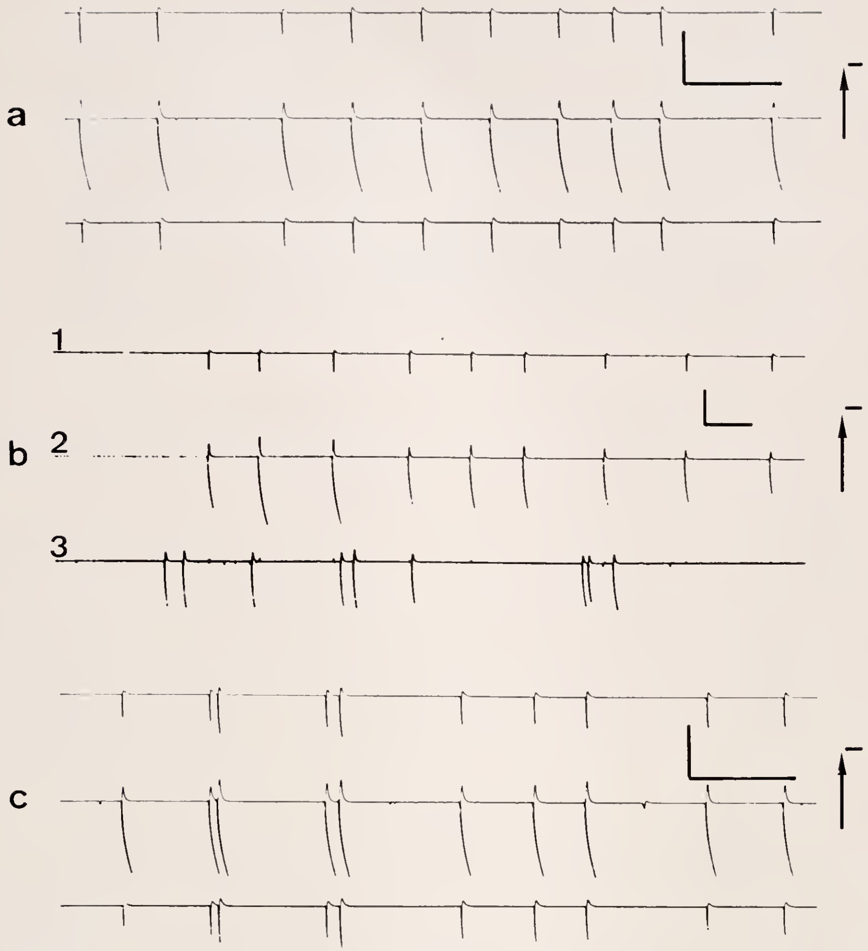

FIGURE 5. a) Synchronous marginal pulse (MP) activity recorded by electrodes attached to three of the four tentacles (perradial) of a recently released medusa. b) MP activity of the same medusa recorded by two electrodes attached to perradial tentacles (traces 1 and 2) and the third electrode (trace 3) attached to a developing interradial tentacles. c) MP activity recorded from the same tentacles as in b) but approximately 20 hr later, with all three sites showing synchrony. In a, b, and c the horizontal bar represents 5 sec and the vertical bar, 1 mV.

related in bursts from different sites. Synchrony of MP's in all the tentacles of free medusae is not the rule; quite frequently populations of tentacles adjacent to one another show connectivity, with each population apparently isolated.

If medusae, both attached and free, are placed in seawater containing excess Mg^{++} then the synchrony of MP's is lost, with all marginal pulse sites eventually becoming silent. If, for example, a mature medusa is placed in a one to one solution of isotonic magnesium chloride and seawater, MP activity ceases after 10 min. This effect is reversible if jellyfish are flushed with fresh seawater, but the rates of recovery are extremely variable.

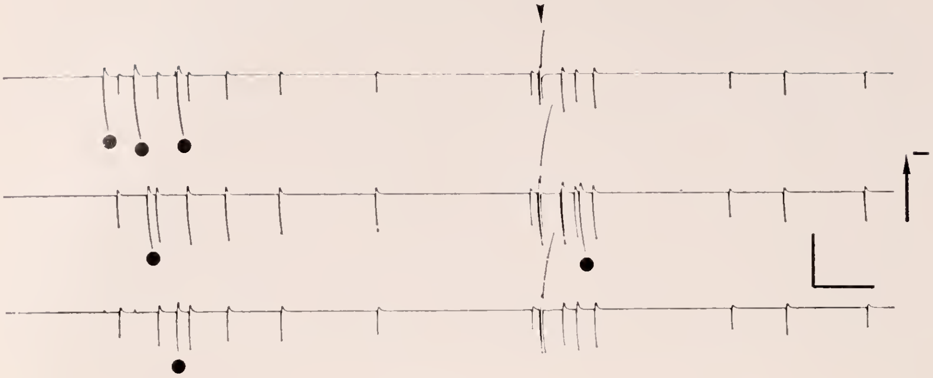

FIGURE 6. Tentacle contraction pulses (TCP's), indicated by solid circles, and marginal pulses (MP's) recorded from three tentacle bases of a medusa attached to the colony. The biphasic event appearing at the arrow on all three channels is a spontaneous crumpling pulse. The horizontal bar represents 5 sec and the vertical bar, 500 μV.

Tentacle contraction pulses (TCP's). These pulses are easily confused with marginal pulses since both pulse types can be recorded from tentacles and have similar parameters. In any one preparation, TCP's normally have a greater amplitude than MP's recorded by the same electrode (Fig. 6), and in multi-channel recordings, TCP's are never synchronized between tentacles. Each tentacle contraction pulse is a local event and can be related to a single unilateral contraction of the longitudinal muscle of the tentacle from which the recording is being made.

Crumpling pulses (CrP's). Suction electrodes can record CrP's from any part of the ectoderm of the medusa. These pulses can be initiated by electrical or mechanical stimulation. Electrical stimulation can elicit a CrP anywhere on the surface of the jellyfish, although greater potentials and larger electrodes are needed to excite the epithelium of the exumbrella than that of marginal regions.

Every crumpling pulse is conducted without decrement over the whole surface of the jellyfish and causes contraction of the smooth, radial muscles of the subumbrella endodermal lamella, and similar muscles in the tentacles. Such contractions are graded, each successive CrP delivered at short intervals (more than 1 shock/4 sec) causes a contraction that summates with prior contractions. There is no evidence of facilitation in the CrP conducting system. In fact, the threshold for evoking a CrP increases with repetitive stimulation. The first shock always causes a CrP, and such a pulse, like all other CrP's is conducted over the entire surface of the jellyfish with a resultant crumple.

The conduction velocity of CrP's may be dependent on the size and geometry of cells forming the ectoderm at any one point, since velocities are greatest in the direction of the long axes of cells and where cells are large (Spencer, 1971). Crumpling pulses that are conducted circumferentially through the exumbrella epithelium close to the margin (within 40 μm) have far higher velocities (mean of 21 cm/sec at 14° C, $N = 52$) than CrP's recorded from other areas of the exumbrella (mean of 13.7 cm/sec at 14° C, $N = 76$).

Recordings of repetitive stimulation of the CrP's system show that the amplitude of pulses decays exponentially to a constant level. This occurs when the inter-

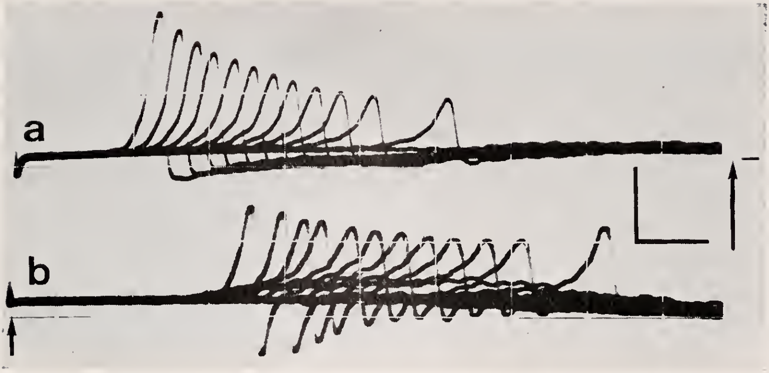

FIGURE 7. Crumpling pulses (CrP's) stimulated at a frequency of 2/sec on the exumbrella and recorded by electrode a) attached to the exumbrella and b) attached to the subumbrella. 12 superimposed traces are shown. An arrow denotes the stimulus artifact. The horizontal bar represents 20 msec and the vertical bar, 1 mV.

val between shocks is less than 1 sec (Fig. 7). During repetitive stimulation at frequencies greater than 1 shock/2 sec the conduction velocity of crumpling pulses decreases with each successive shock until the system becomes inexcitable. This phenomenon will be called fatiguing. All parts of the ectoderm show fatiguing to the passage of closely spaced crumpling pulses, and since the phenomenon of fatiguing can be seen in preparations where the duration of the shock is less than 1 msec, increased initiation delay cannot account for fatiguing. Recovery from a fatigued state is fairly rapid with the conduction velocity returning to 90% of the original value in about 40 sec.

DISCUSSION

The feeding pattern seen in *P. flavicirrata* closely follows feeding habits in other hydromedusae. Hyman (1940) distinguishes between feeding in jellyfish having a broad, shallow bell and those having a tall, narrow bell. These characteristics are typical of the leptomedusae and anthomedusae respectively. Feeding in *P. flavicirrata* most closely resembles that of the flat type of jellyfish, except that tentacles holding the food contract and these tentacles are sometimes held by the manubrial lips. Low amplitude potentials have been recorded from the bases of perradial tentacles involved in a feeding response that cannot be associated with other known conduction systems (Spencer, 1971).

The only analytical accounts of the mechanics of swimming in the hydromedusae are those given by Gladfelter (1972, 1973). All the principles of propulsion he suggests are met by *Proboscidactyla* except that velar steering was not seen. Unlike other limnomedusae *Proboscidactyla* does not have statocysts and it is likely that the extra stability gained from a dome-shaped bell obviates the need for a reflex response such as velar steering. Synchronous tentacle contractions accompanying or preceding swimming have been frequently seen in hydromedusae; *Eperetmus typus* (Mackie and Mackie, 1963), *Leuckartiara octona* (Russell, 1953), *Bougainvillia superciliaris* (Agassiz, 1849). Such contractions presumably reduce the drag created by trailing tentacles.

Spontaneous swimming in scyphozoans (Horridge, 1959) is unlike that in small antho- and limnomedusae. Bursting is rarely seen and there is far more variability in the mean interpulse interval over extended periods of time. Horridge found that the standard deviation of the inter-contraction interval is usually 20–30% of the mean, whereas in *P. flavicirrata* equivalent values are from 4–21% (calculated from the raw data for Table II).

Behavioral and electrophysiological evidence (Romanes, 1876; Passano, Mackie and Pavans de Ceccatty, 1967) suggests the presence of a number of coupled swimming pacemakers in the marginal ganglia (Mackie, 1971) and/or marginal nerves of hydromedusae. Mackie and Passano (1968) have shown that the spread of excitation for contraction in the swimming, circular muscle of *Sarsia* does not require the involvement of nerves. Pure myoid conduction in *Sarsia* has a velocity of 7.5–8.5 cm/sec (20° C) in a circular direction. If the margin is present then velocities rise to 50 cm/sec as compared to 25 cm/sec (14° C) in *Proboscidactyla*. It is possible that in *Proboscidactyla* circular conduction is facilitated by the presence of the marginal nerve rings (Spencer, 1971). Thus in many hydromedusae the marginal nerves are used for rapid conduction of excitation around the bell and the swimming-muscle sheet is excited near the margin by these nerves with a subsequent myoid spread.

The spikes (PSP's) sometimes seen in *Proboscidactyla* preceding biphasic swimming pulses and during recovery from Mg^{++} anaesthesia are presumably the motor output of the swimming pacemaker system, conducted in the marginal nerve or nerves that trigger swimming. Similar events have been recorded in *Phialidium* (Passano, 1965), *Spirocodon* (Ohtsu and Yoshida, 1973), and *Stomotoca* (Mackie, unpublished). Since PSP's can be recorded in the margin before the swimming muscle has recovered from Mg^{++} anaesthesia it would appear that Mg^{++} blocks transmission at neuro-muscular junctions more readily than neuro-neuronal junctions.

Spontaneous, synchronized tentacle flexion or shortening is seen in many hydrozoans, both polyps and medusae: *Porpita* (Mackie, 1959), *Tubularia* (Josephson, 1965), *Turritopsis* and *Gossea* (Gosse, 1853), and *Eperetmus* (Mackie and Mackie, 1963). The functions of such movements are obscure in *Proboscidactyla* as in other hydromedusae. In *Porpita,* orally directed flexions seem to aid food collection whereas in *Tubularia* similar movements are probably used to mix the contents of the proboscis cavity and/or to transport gut contents to neighboring polyps.

Marginal pulses always accompany synchronized tentacle contraction in *Proboscidactyla* although marginal pulse firing does not necessarily cause tentacles to contract. Passano *et al.* (1967) report that synchronous contraction is seen during MP firing but that, unlike *Proboscidactyla*, there is not a one for one relationship. No motor function has been ascribed to MP's recorded from *Spirocodon* (Ohtsu and Yoshida, 1973); I assume the nMP's they report to be synonymous with MP's, and that QSCP's are the electrical concomitant of the type of tentacle contraction that can be triggered by nMP's. It is evident that MP's can only be recorded from areas close to the margin and that the conduction system lies within the marginal nerve rings (Passano, 1965; Passano *et al.,* 1967; Mackie and Passano, 1968; Ohtsu and Yoshida, 1973). There is a striking difference between the conduction velocity of marginal pulses in *Sarsia* (50 cm/sec) and *Spirocodon*

(71 cm/sec) with the velocity in *Proboscidactyla* (4.7 cm/sec). An explanation for this difference is that the diameters of neurons conducting MP's are likely to be far smaller than equivalent neurons in *Sarsia* and *Spirocodon* since the individuals used were young attached medusae.

The ontogeny of activity in the marginal pulse system suggests that every tentacle has the potential to contain a marginal pulse pacemaker and that synchrony between MP pacemaker sites is dependent on neuronal connections between sites. These connections probably consist of a number of parallel pathways, thus affording either tight or loose coupling of pacemakers. This coupling is lost under Mg^{++} anaesthesia before the pacemakers become completely silent (*cf.* Mackie and Passano, 1968).

Romanes (1877) was familiar with crumpling in jellyfish and gives a detailed description of a "spasm" in *Staurophora,* noting all those features which distinguish it from a swimming contraction. Unfortunately he was not aware of the existence of a second muscle system, the radial muscles. Mackie, Passano and Pavans de Ceccatty (1967) did identify the muscles involved and give a description of the events occurring after excitation of the exumbrella of *Sarsia tubulosa.* In *Sarsia* crumpling consists of a flurry of contractions in the radial muscles, and closure of a sphincter in the bell margin whereas in *Proboscidactyla* there is often just a single contraction and the sphincter muscle is absent. It should be noted that, besides protecting delicate tissues, crumpling causes *Proboscidactyla* to sink more rapidly by reducing the frontal area, thus carrying an individual away from noxious stimulation.

The crumpling pulses that can be recorded from *Proboscidactyla* can be equated with similar potentials that have been recorded in most of the hydrozoans so far examined. Histological evidence from Mackie (1965), Mackie and Passano (1968) and Spencer (1971) shows that these pulses can be propagated across sheets of simple pavement epithelium of either the bell or nectophore (in spihonophores). These epithelia do not appear to support any other electrical activity and the excitation that is propagated from cell to cell causes similar motor activity in all the hydromedusae studied, that is crumpling. The electrical parameters of crumpling pulses are characteristic, being of large amplitude (several mVs) and biphasic. In some species they may be compound events as Passano (1965) has shown in *Cladonema.*

None of the epithelia conducting CrP's show any signs of facilitation, the first shock is always effective. Multiple firing to a single shock seems to be a common feature of the systems in *Sarsia* and *Euphysa* (Mackie and Passano, 1968) unlike *Proboscidactyla* where it never occurs. During such multiple firing the duration of each pulse increases successively, this is similar to an increase in the duration of CrP's in *Proboscidactyla* after repetitive stimulation and maybe consequential to a reduction of conduction velocity as the system fatigues. The conduction velocities of CrP's in *P. flavicirrata* of 6–21 cm/sec at 14° C over the exumbrella ectoderm is of the same order as velocities measured in other hydromedusae; *Sarsia,* 15 cm/sec at 18° C (Mackie and Passano, 1968); *Euphysa,* 18 cm/sec at 14° C (Spencer, 1971); *Bougainvillea,* 6 cm/sec at 14° C (Spencer, 1971); *Phialidium,* 19 cm/sec at 20° C (Mackie and Passano, 1968); and *Spirocodon,* 12 cm/sec (Ohtsu and Yoshida, 1973). Differences in conduction velocity of crumpling pulses due to the geometry of cells in the ectoderm have not been seen in other hydromedusae. The very high velocities of CrP's conducted circumferentially near the margin may be due to facilitated conduction caused by involvement of the marginal nerve rings (Spencer, 1971).

Proboscidactyla is still able to conduct crumpling pulses in excess Mg^{++} (Spencer, 1971) even when all muscular and nervous activity has stopped, though these pulses can only be generated by electrical stimulation. This low susceptibility of epithelial systems to magnesium anaesthesia was noted by Mackie and Passano (1968).

The phenomenon of fatiguing where both amplitude and velocity of epithelial pulses falls with repetitive stimulation has not been recorded in other hydromedusae, though Mackie and Passano (1968) did notice an increase in the duration of crumpling pulses with multiple firing.

The radial muscles responsible for crumpling are smooth muscles but it is not known whether such muscles in hydromedusae require nervous innervation or whether the myoepithelial cells can be depolarized electrically by direct epithelial excitation. Perhaps both types of innervation are present since the graded, local movements seen during prey capture and swallowing obviously require nervous control.

From the physiological and histological evidence obtained from a preceding paper on the polypoid phase of this limnomedusan (Spencer, 1974) and that presented here, some hypotheses on the mechanisms of epithelial conduction in the Hydrozoa can be erected.

We find, as a general rule, that conduction velocities of epithelial pulses are higher in tissues having large cells. For example, crumpling pulses have higher velocities (6–21 cm/sec) than colonial pulses (0.9–9.0 cm/sec) in *Proboscidactyla* and cells of the exumbrella ectoderm are far larger (approximately 25 μm diameter) than the epithelial cells that probably conduct colonial pulses (approximately 12 μm diameter). In *Hippopodius* (Siphonophora) the exumbrella cells of the nectophores are often 100 μm on their shortest axis (Mackie, 1965) and have conducted pulses with velocities as high as 30 cm/sec at 21° C. This suggests that a large component of the conduction time of an epithelial pulse may be due to a delay at each cell-junction. It should be noted that all these cells have similar thicknesses and thus a lower cytoplasmic resistance probably does not account for such high velocities in the larger cells.

The phenomenon of fatiguing in the epithelial conducting systems of *Proboscidactyla* gives further support to the idea that junctional delay may be a prime factor in determining conduction velocity. During fatigue of the colonial pulse system of the polyp colony, to repetitive stimuli, the velocity of pulses may be reduced by 45% of the original velocity but there is no reduction in amplitude, whereas in the crumpling pulse system of the medusa the velocity is maximally reduced by 40% and there is an exponential decrease in the amplitude of pulses. This decrease in conduction velocity of epithelial pulses seen during repetitive firing could be due to: a) an increase in the thereshold of each cell, b) an increase in the rise time of the action potential, or c) an increase in the proportion of inexcitable cells, that is cells not recovered from their refractoriness. There is no reason why these mechanisms should be mutually exclusive.

I am grateful to Dr. G. O. Mackie for his guidance and enthusiasm throughout this study. This work was supported by an operating grant to G. O. Mackie from the National Research Council of Canada and by an NRC graduate scholarship to the author. This paper is based on part of a thesis submitted by A.N.S. to the Department of Biology, University of Victoria, in partial fulfillment of the requirements for the degree of Ph.D.

SUMMARY

1. Feeding in the medusa of *Proboscidactyla flavicirrata* is accompanied by local, small amplitude impulses recorded from the bases of perradial tentacles.

2. Medusae, both attached and free, swim spontaneously with electrodes in place thus giving data on the temporal patterns of contractions of the swimming muscle.

3. Swimming pulses (SP's) can be recorded from the circular muscle of the subumbrella. Each SP is preceded by a pre-swim pulse (PSP) which is a neuronal pulse conducted in the marginal nerve(s).

4. Marginal pulses (MP's) are neuronal pulses conducted in the marginal nerve(s) which can trigger synchronous tentacle contraction.

5. MP's originate from pacemaker sites located in tentacle bulbs. When new tentacles first appear the firing of MP's from these new sites is not synchronized with established pacemakers: eventually they become linked to the original pacemaker system.

6. Synchrony between MP pacemakers is lost under Mg^{++} anaesthesia.

7. Tentacle contraction pulses (TCP's) are the electrical accompaniment to local tentacle contraction.

8. Crumpling pulses (CrP's) are epithelial pulses causing the protective behavior known as crumpling.

9. CrP's show a decrease in conduction velocity and amplitude as a result of repetitive stimulation.

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THE ONTOGENY AND SPECTRAL SENSITIVITY OF POLAROTAXIS IN LARVAE OF THE CRAB *RHITHROPANOPEUS HARRISI* (GOULD)

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Both terrestrial and aquatic arthropods have been shown to possess polarization sensitivity (Eguchi and Waterman, 1968; Shaw, 1966, 1969b; Snyder, Menzel and Laughlin, 1973; Waterman, Fernandez and Goldsmith, 1969; Waterman and Horch, 1966) and frequently stereotyped polarotaxes are displayed (reviewed, Waterman, 1974). Among crustaceans the ontogeny of polarotaxis during larval development is unknown, since nearly all experiments on polarized light perception have concerned adults. In these experiments, we have studied polarotaxis in the planktonic larvae of an intertidal crustacean.

Because planktonic organisms are small and can travel rapidly over short distances, difficulties in monitoring their movements accurately and conveniently have limited their use, a problem which is augmented for larval forms by factors reducing success in rearing them to advanced stages in the laboratory. With a closed circuit television system combining real-time recording and slow motion playback, we have been able to accurately monitor the swimming directions in response to polarized light of larvae of the low intertidal crab *Rhithropanopeus harrisi.* These larvae are especially suited for such a study, since they are good swimmers and their phototactic responsiveness to light is well described (Forward, 1974; Forward and Costlow, 1974). In addition, Costlow, Bookhout and Monroe (1966) have characterized the developmental sequence and established extensive rearing techniques, thereby eliminating many potential difficulties in raising larvae for these experiments.

Results of our examination of the ontogeny of polarotaxis in *R. harrisi* indicate that only zoeal stages II and III respond to polarized light. Consideration of the intensity and spectral characteristics of the response reveals that maximal responsiveness occurs at an intensity of 10^{-2} W/m^2 and at 500 nm. Larvae are strongly phototactic and when intensity distributions and polarization are offered antagonistically, they respond with nearly equal phototaxis and polarotaxis. Following dark adaptation, the polarotactic response disappears.

Materials and Methods

Experimental animal

Ovigerous females of the low intertidal crab *Rhithropanopeus harrisi* were allowed to hatch under controlled conditions: salinity, 20‰; L:D cycle, 12:12; temperature cycle, 20–25° C. The experiments described in the ontogeny section were performed on larvae of crabs procured in Florida, while crabs collected locally were utilized in the experimental determination of the wavelength sensitivity. After hatching, larvae were changed daily to fresh filtered 20‰ seawater, fed newly hatched *Artemia* nauplii, and maintained under a 12:12 L:D cycle at a constant temperature of 25° C. These conditions were selected because previous work has indicated that they produce maximum developmental success (Costlow *et al.*, 1966).

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Apparatus

The arrangement of the basic apparatus is depicted in Figure 1. A slide projector with a tungsten lamp acted as the stimulus source. Light passing through the projector optics was filtered by two hot mirrors (Baird Atomic), and two Corning No. 1–75 filters to remove heat, plus selected neutral density filters (Oriel Corporation of America). Wavelength was controlled by either an interference filter (Optics Technology, λ's 453, 470, 499, 556, 620 nm; half band pass 3–5% of wavelength), or a thin film absorption filter (Ditric Optics Inc., λ's 420 nm, half band pass 7 nm; 520 nm, half band pass 8 nm; 600 nm, half band pass 10 nm). A 300 W lamp was used for stimulus intensities below 0.1 W/m^2, while a 750 W lamp was used for a higher intensity. Intensities were measured with a radiometer (Yellow Springs Instruments, Model #65).

Light thus filtered first passed through two layers of waxed paper to depolarize any polarized light emitted from the source and then was linearly polarized by a polaroid sheet (Carolina Biological Supply.) The advantage of using this depolarizer/polarizer sandwich is that uniformly unpolarized light of approximately the same intensity as the polarized test light could be provided as the stimulus for control experiments by turning the waxed paper to the underneath side. The polaroid was mounted in the center of a rotatable circular mask. Horizontal shielding was provided directly above the experimental vessel in the form of a circular mask overhanging the sides by 1 cm. This eliminated potential interaction of the light with the sides of the vessel, a clear plastic dish (diameter 8.5 cm). To minimize other possible sources of scattering, all baffling surfaces were painted flat black, and the room was completely darkened for experimentation.

Responses were monitored by a video camera (Cohu Model 4300) positioned directly below the vessel. Circular baffles extending downward around the lens from the glass sheet on which the vessel rested insured that the only visible portion of the camera was the lens itself. The video picture was viewed on an Electrohome monitor (Model #EVM–11), and recorded on a Panasonic video tape recorder (#NU–3020 SD). Time markers were entered on the tape by voice commands.

Analysis and statistical treatment of data

In analysis, the tapes were replayed at slow speeds, and the swimming path of each animal visible within a given time period was traced onto a Plexiglas sheet fitted over the monitor face. Since the animals are small and can frequently change swimming direction, a criterion had to be devised to standardize the portion of the path chosen for measurement, and so it was decided to measure the swimming direction of the first straight segment longer than 1.5 cm on the Plexiglas sheet within a given time interval after the onset of stimulation. This technique is similar to that previously employed by other investigators (*e.g.*, Bainbridge and Waterman, 1957; Umminger, 1968). In all experiments 150–200 individual paths were measured, and final distributions represent an average of larvae from 3–6 different hatches.

The compass direction of each path was measured with a protractor and recorded in 10° sectors centering on 0°, 10°, etc., through 350°. 0° was arbitrarily established and served as a reference direction for swimming paths and polaroid alignment.

Under polarized light, four angles of the polaroid relative to the 0° reference (0°, 50°, 90°, 140°) were tested to minimize possible effects of intensity artifacts within the experimental vessel. Then, to combine these data into one angular distribution of swimming paths relative to the angle of polarization, the distributions under each polarization condition were transposed and plotted relative to the e-vector placed at 0°. Since within the experimental situation, the 0° direction is identical to 180°, and 10° to 190°, etc., the data at these angles were combined to produce an angular distribution showing swimming directions between 0° and 170° with the e-vector at 0°.

Chi squares were computed for the resulting distributions, and a significance level of 0.05 was used as the cutoff for a non-random distribution. The significance of orientation at certain angles as compared to the distribution mean was further checked using the McNemar test for significant changes. To standardize the graphs of orientation angle versus directional frequency, the percentage of the total number of paths that occur at a particular angle are plotted rather than the actual number of paths.

Experimental procedure

The responses of both light and dark adapted animals to different stimulus intensities were first-tested. Animals were light adapted under room lights plus a 60 W incandescent lamp placed directly over the bowls. All experiments were performed on the second day of the three-day molt cycle of zoeal stages I–IV. The megalopa were not tested because preliminary experiments indicated that they rarely move upon stimulation. To avoid variations in responsiveness due to a possible biological rhythm, experiments were always performed between two and six hours after the onset of the light phase.

Approximately 50 larvae were placed in the vessel which was then positioned above the camera and filled to the brim with water to eliminate the differential reflection effects of a meniscus. The plastic experimental vessel may have differentially transmitted polarized light and thereby provided an orientation cue. To reduce the possible effects of this cue, the dish was randomly oriented relative to the e-vector direction for each trial. The larvae were maintained in darkness for 15 seconds and then stimulated for 15 seconds with various intensities of 499 nm light. This wavelength was selected because it is the primary maximum in the sensitivity spectrum for phototaxis in *R. harrisi* (Forward and Costlow, 1974).

Swimming paths were recorded from 10–15 seconds after the initiation of the stimulus, since preliminary trials demonstrated consistent responsiveness during this time interval. Then, after the animals had rested in darkness for 15 seconds, a second stimulus was presented and responses recorded similarly. At a given intensity, four angles of the polaroid were tested, but to preclude fatigue effects, each group of larvae was used for only one angle, that is, one set of two stimuli Although repetitive runs were usually necessary, there were always at least 15 minutes between reuse of any larvae, and upon reuse, the animals were exposed to a different angle of polarization and/or stimulus intensity. Controls were performed in the same sequence by simply reversing the polaroid/depolarized sandwich so that the animals were exposed to unpolarized light of the same intensity and angular distribution.

Dark adaptation was accomplished by placing larvae in experimental vessels on a table beneath a lightproof cover for a minimum of 1½ hours prior to the beginning of testing. This preplacement eliminated the necessity for subsequent visual transfer under light. Any light then needed for examination was interference filtered to 600 nm, since larvae are largely phototactically unresponsive to this wavelength (Forward and Costlow, 1974). Since light-adapted stage II larvae proved to be the most responsive to polarized light only these were tested in the dark adaptation experiments. To avoid excessive light adaptation, responses were recorded for the interval 0–5 seconds after stimulus initiation, and the time between stimuli was lengthened from 15 to 30 seconds. Although the stimuli for these experiments were shorter than those used in light adaptation, preliminary experiments showed that this interval was satisfactory to maintain dark adaptation and to initiate directional swimming.

Since stage II larvae are most responsive to polarized light, their spectral sensitivity for polarotaxis was tested between 420 to 620-nm light. At 499 nm, an intensity of 2.5×10^{16} quanta/m^2/sec (10^{-2} W/m^2) produced the greatest response. Thus, quantal intensities for other wavelengths were adjusted to this value ($\pm 9\%$) by means of neutral density filters. The experimental sequence was basically the same as in the previous experiments. Four angles of the polaroid were tested for each wavelength, with two stimuli per preparation. Responses were again recorded during the interval 10–15 seconds after the initiation of the stimulus, and larvae of each hatch were examined at all wavelengths. The data from three hatches were grouped and the percent swimming perpendicular to the e-vector computed for each wavelength.

To determine that the observed polarotaxis does not result from phototaxis to the intensity distribution pattern created by polarized light, experiments with light-adapted stage II larvae were performed in which phototaxis was competitively tested against polarotaxis. Phototaxis was established by recording swimming paths in unpolarized light with a collar of alternating black and white sectors each subtending an angle of 90° surrounding the vessel. Phototactic sign was determined by grouping swimming paths into those toward the white (+) and those toward the dark (−). An antagonism between phototaxis and polarotaxis was then produced under polarized light by aligning the e-vector parallel to the white sectors. Thus, animals responding to the e-vector should swim perpendicularly into the black sectors, while at the same time animals should also be drawn phototactically to the white sectors. For both experiments, the stimulation and recording procedure was the same as described previously for light-adapted larvae.

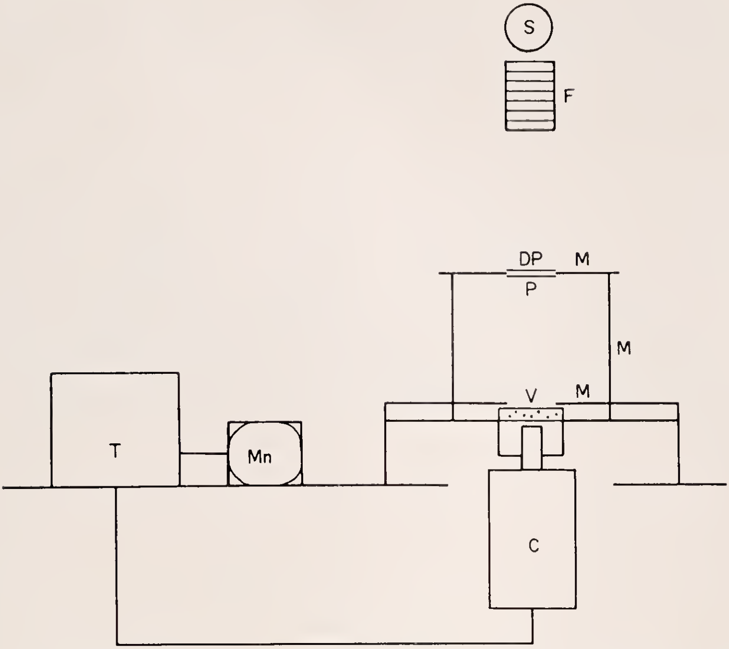

FIGURE 1. Experimental apparatus. The various components are tungsten light source (S), filter holder (F), depolaroid/polaroid (DP/P), masks painted flat black (M), experimental vessel (V), television camera (C), video monitor (MN), video tape recorder (T).

RESULTS

Ontogeny of polarotaxis

Light-adapted larvae of *R. harrisi* can show a definite response to polarized light which is not only non-random, but is stereotyped within a given stage as to the preferential swimming direction in relation to the polarization plane. In our experiments, however, not all stages were responsive, and even among those which showed significant non-random swimming, the strength and angular maximum of the response varied.

For stage I (Fig. 2) none of the angular distributions for any of the test intensities differ significantly from a random distribution nor do any of the angles have a number of swimming paths that differ significantly from the mean. In contrast, stage II (Fig. 3) shows strong swimming orientation perpendicular to the e-vector with the strongest response (19.5%) occurring at a stimulus intensity of 10^{-2} W/m^2 (Fig. 3c). Stage III (Fig. 4) exhibited a weaker response, as well as a change in the preferred swimming direction. At intensities of 10^{-1} and 10^{-2} W/m^2 significant swimming occurred parallel to the e-vector. Stage IV (Fig. 5) shows no orientation to polarized light, as none of the angular distributions at different stimulus intensities differs from a random distribution. Swimming under unpolarized light in the control experiment was random in all stages and at all intensities (Table I).

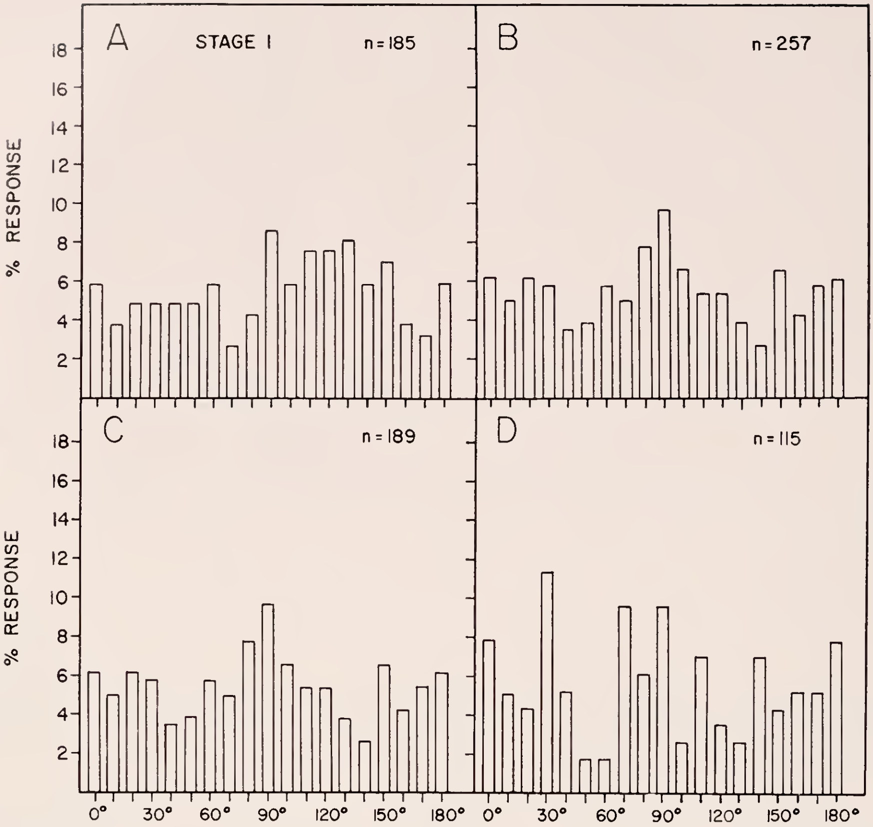

FIGURE 2. Angular distributions of swimming directions in stage I zoeae under linearly polarized light at 499 nm at intensities of 2×10^{-1} W/m^2 (A), 10^{-1} W/m^2 (B), 10^{-2} W/m^2 (C), and 10^{-3} W/m^2 (D). For symmetry, the percent response at 0° has been replotted at 180°. The χ^2 for the overall distribution at each intensity (A. 16.697, B. 10.760, C. 14.450, and D. 26.651) indicates none of the distributions differ significantly from random. In all figures, if the number of swimming paths at a particular angle is significantly different from the mean, one asterisk indicates $P \leq 0.05$ and two asterisks indicate $P \leq 0.005$.

Stimulus intensity had a pronounced effect on the response. The highest intensity tested, 2×10^{-1} W/m^2, was apparently above the upper intensity threshold for polarotaxis, as it evoked random swimming in all stages. In both stages II and III, responsiveness increased at lower intensities to a maximum at 10^{-2} W/m^2, and then disappeared entirely at the very dim 2×10^{-4} W/m^2. From Figures 3 and 4, the upper intensity threshold for polarotaxis by stages II and III can be placed between 1×10^{-1} and 2×10^{-1} W/m^2, and the lower threshold between 2×10^{-4} and 1×10^{-3} W/m^2.

The typical perpendicular orientation seen in light-adapted stage II larvae was absent subsequent to dark adaptation (Fig. 6). Although a non-random angular distribution did occur at 10^{-2} W/m^2 (Fig. 6c), there are no significant maxima.

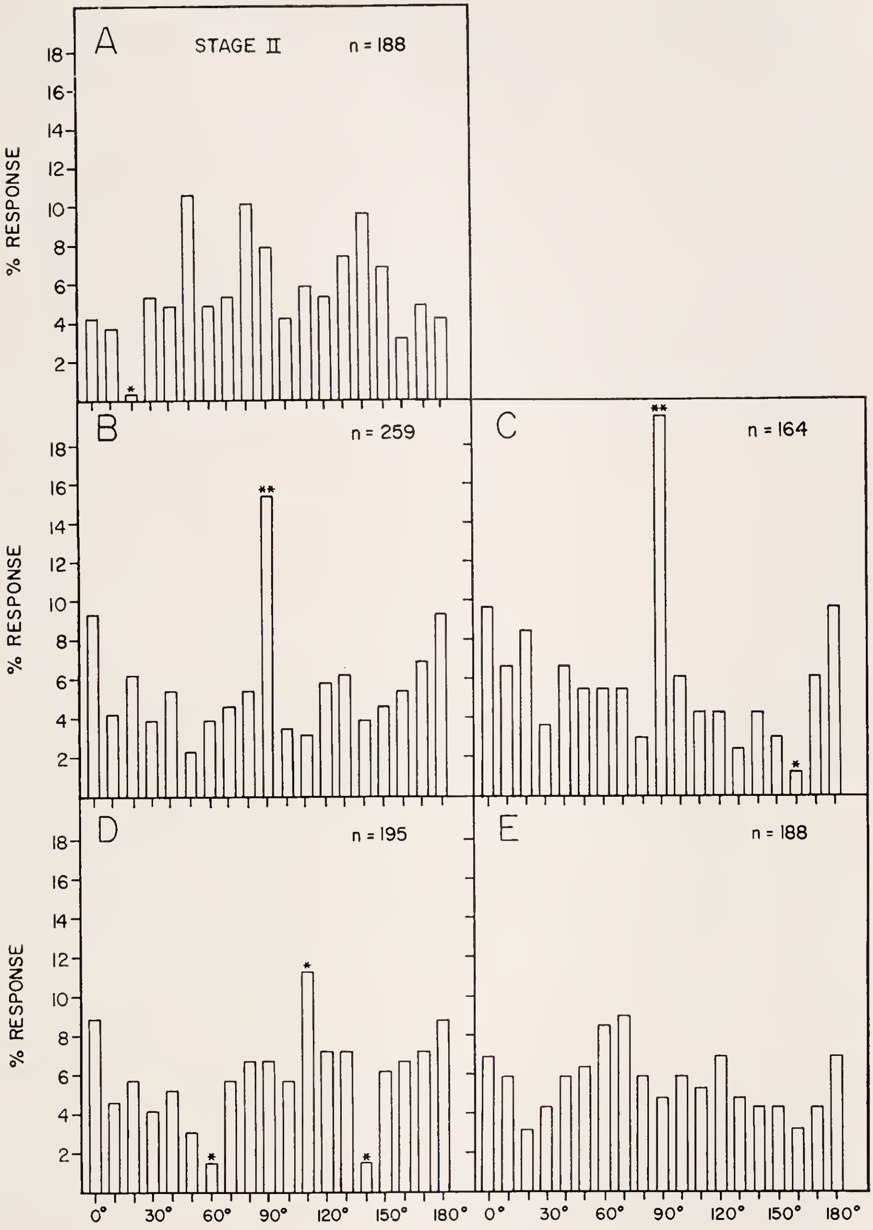

FIGURE 3. Angular distributions of swimming directions in stage II zoeae under polarized light at 499 nm at intensities of 2 × 10^{-1} W/m^2 (A), 1 × 10^{-1} W/m^2 (B), 10^{-2} W/m^2 (C), 10^{-3} W/m^2 (D) and 2 × 10^{-4} W/m^2 (E). Treatment and presentation of data are the same as in Figure 2. The χ^2 values (A. 36.349, B. 68.682, C. 80.949, D. 31.75, E. 11.752) indicate that all distributions except E differ significantly from random.

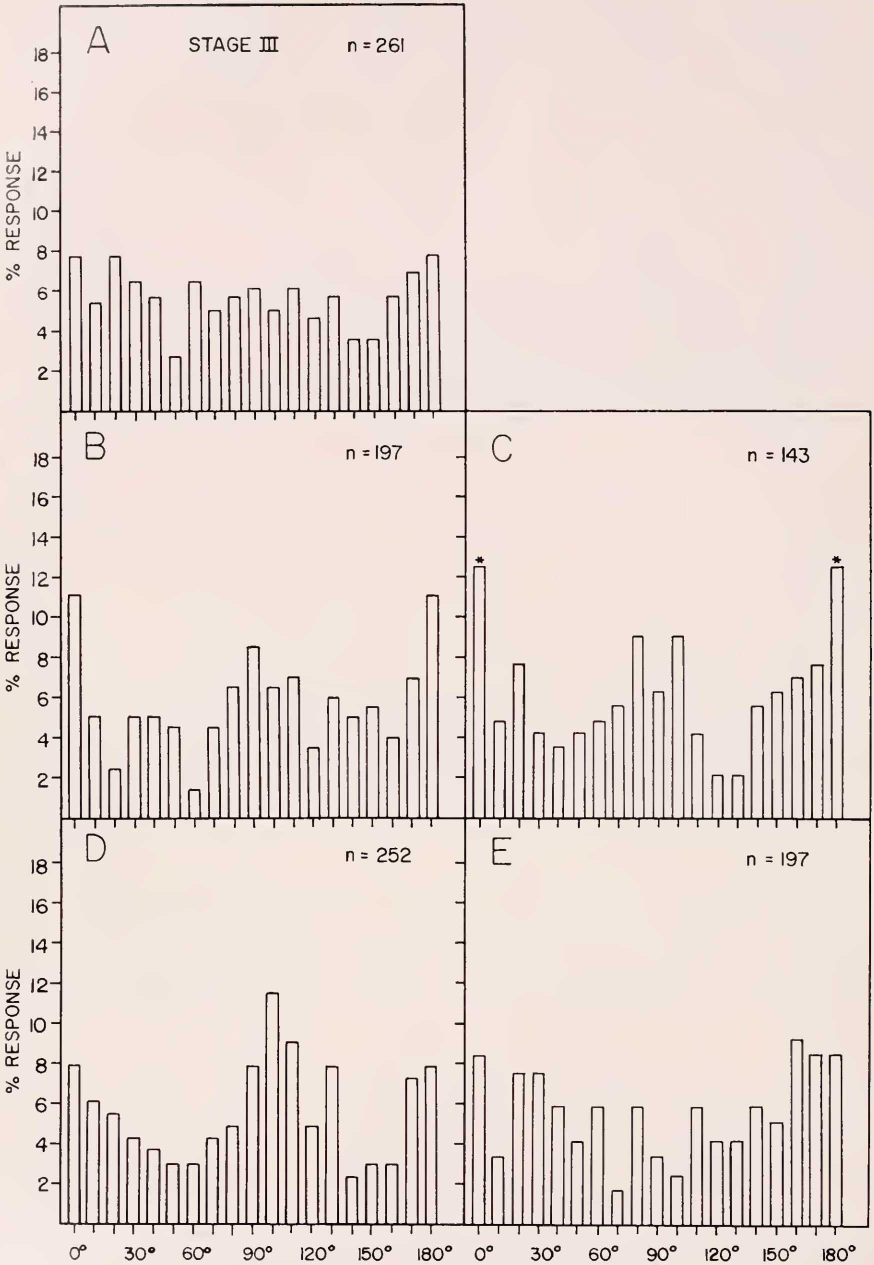

FIGURE 4. Angular distributions of swimming directions in stage III zoeae under polarized light at 499 nm at intensities of 2×10^{-1} W/m² (A), 10^{-1} W/m² (B), 10^{-2} W/m² (C), 10^{-3} W/m² (D) and 2×10^{-4} W/m² (E). Treatment and presentation of data are the same as in Figure 2. The χ^2 for the overall distributions (A. 15.067, B. 29.174, C. 28.004, D. 32.985, and E. 15.037) indicate only those in B, C and D differ significantly from random.

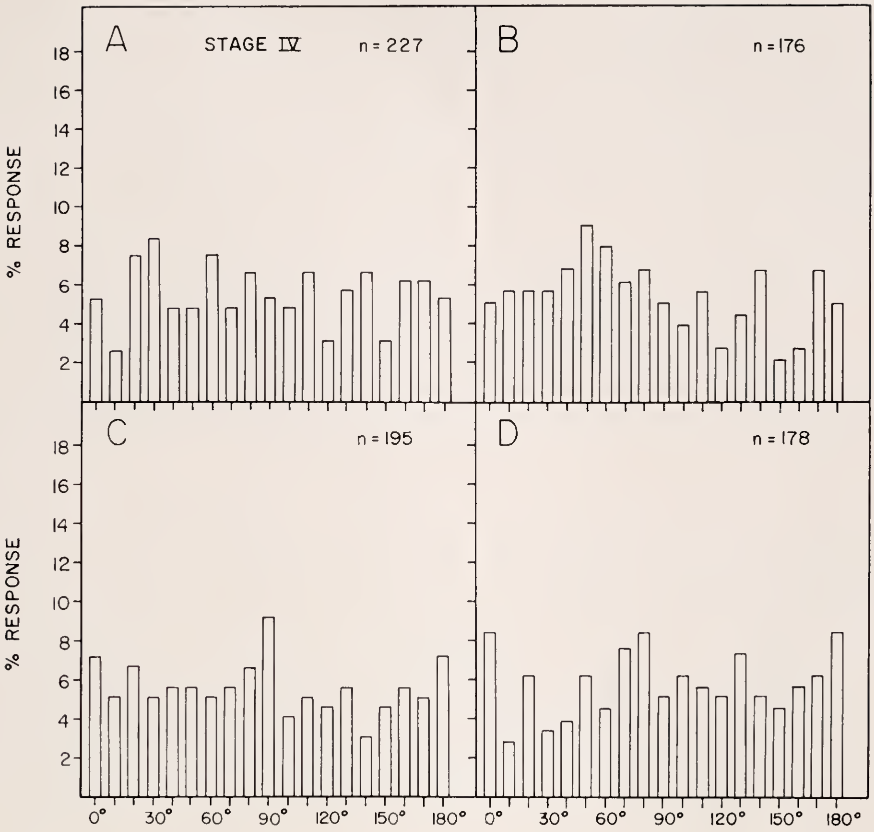

Figure 5. Angular distributions of swimming directions in stage IV zoeae under polarized light at 499 nm at intensities of 2×10^{-1} W/m² (A), 10^{-1} W/m² (B), 10^{-2} W/m² (C) and 10^{-3} W/m² (D). Treatment and presentation of data are the same as in Figure 2. The χ^2 values for the overall distributions (A. 17.130, B. 14.898, C. 9.640, and D. 12.357) indicate none of the distributions differ from random.

Table I

χ^2 values for swimming distributions under depolarized light in control experiments.

	Stimulus intensity (W/m²)			
	10^{-3}	10^{-2}	10^{-1}	2×10^{-1}
Stage I	20.843	23.773	26.150	13.881
Stage II	—	12.724	9.245	15.941
Stage III	19.517	16.312	22.727	13.683
Stage IV	11.407	17.271	14.077	15.014
Dark adapted stage II	18.992	31.993	14.479	15.894

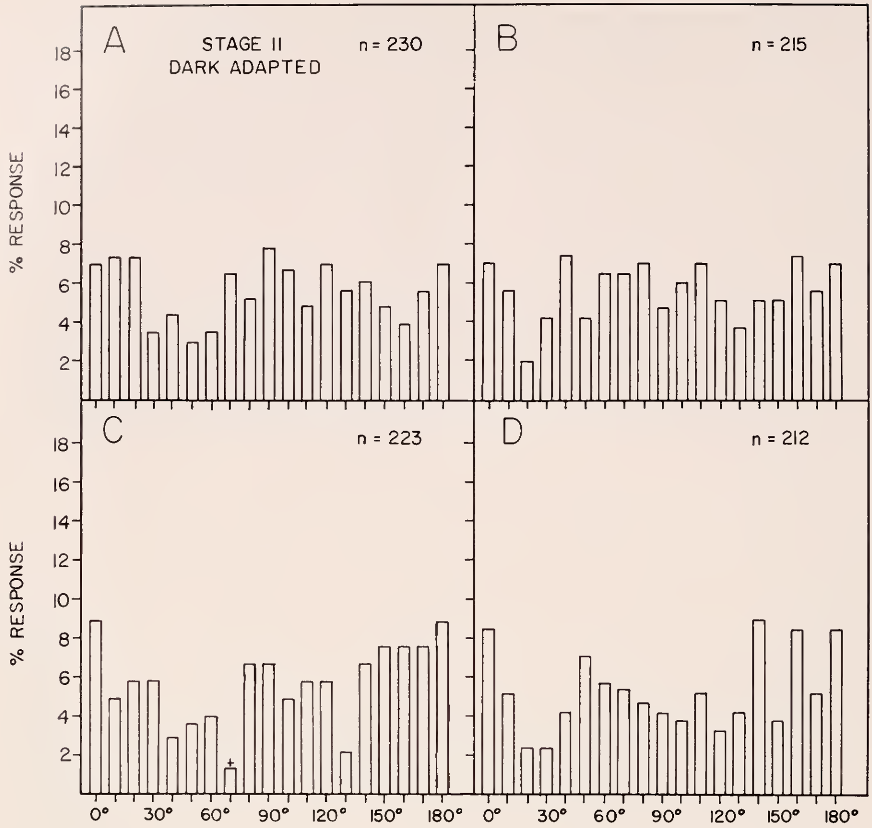

FIGURE 6. Angular distributions of swimming directions for dark adapted stage II zoeae under polarized light at 499 nm of intensities 2×10^{-1} W/m^2 (A), 10^{-1} W/m^2 (B), 10^{-2} W/m^2 (C) and 10^{-3} W/m^2 (D). Treatment and presentation of data are the same as in Figure 2. The overall distributions in A ($\chi^2 = 15.984$), B ($\chi^2 = 14.479$) and D ($\chi^2 = 18.992$) do not differ from random while that in C ($\chi^2 = 31.993$) is significantly different ($P < 2.5\%$).

Spectral sensitivity

When tested under various wavelengths, stage II larvae from females collected locally showed maximum responsiveness to 499-nm light (Fig. 7). In fact, this is the only wavelength at which significant ($P < 0.005$) perpendicular swimming occurred. The angular distribution for 420 nm was significantly different from a random distribution ($P < 0.05$) but the 9.7% response at 90° was not significant. This suggests that another maximum in the near ultraviolet might become apparent if shorter wavelengths could be tested. For all other wavelengths, the angular distributions were random, and the percent response fell close to the expected 5.5% for totally random swimming. It should be noted that the responsiveness of these larvae was 11.8% at 499 nm, as opposed to the 19.5% shown under the same experimental conditions by the larvae from crabs from Florida (Fig. 3c). This difference may indicate that responses may vary between populations.

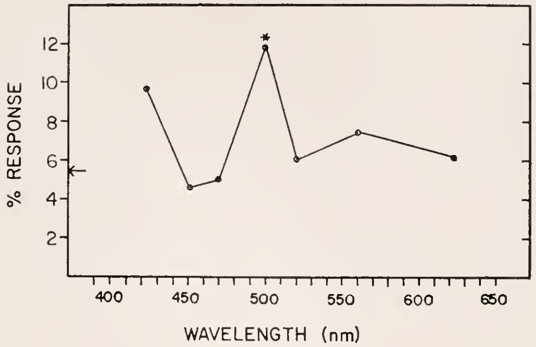

FIGURE 7. Spectral sensitivity for polarotaxis. Points represent the percentage of swimming perpendicular to the e-vector (ordinate) at the stimulus wavelengths (abscissa). The horizontal arrow shows the expected percentage response for random swimming.

Phototaxis vs. *polarotaxis*

In these tests the experimental vessel was surrounded by a collar of alternating black and white quadrants. Under depolarized light, a significantly greater ($P < 0.005$) number of light adapted stage II larvae (62%) swam in the direction of the light quadrants than toward the dark quadrants of the experimental vessel (36%). Under polarized light with the e-vector parallel to the white quadrants, this positive phototaxis was reduced since nearly equal numbers of animals swam toward the dark quadrants (48%) as toward the white quadrants (52%). In this experimental situation the centers of the dark quadrants are perpendicular to the e-vector which is the dominant polarotactic orientation direction for stage II larvae. Since positive phototaxis is reduced, this suggests that a polarotactic response which is independent of phototaxis contributes to directional swimming.

DISCUSSION

It is clear from these results that marked changes occur in polarotaxis during the larval development of the crab *Rhithropanopeus harrisi.* While stage II and III zoeae are significantly polarotactic (stage II perpendicular and stage III parallel to the e-vector), larvae of stages I and IV are unresponsive. Because polarized light perception is a function of precision in anatomical dimensions, the onset of polarotaxis in stage II may result from functional alterations associated with the morphological change between the sessile eyes of stage I and the stalked eyes of stage II (see Costlow and Bookhout, 1971, for diagram). There are no such externally obvious changes between stages III and IV which could account for the altered responsiveness there, however, only an ultrastructural examination could reveal definitively what dimensional changes occur within the eye. Responsiveness may also be altered by developmental changes in higher order neural anatomy.

Both the magnitude and the direction of the response of *R. harrisi* larvae are in good agreement with previous studies on adults of other crustacean species. Considering the absolute percent response at a particular angle of orientation as opposed to the "degree of orientation" [defined as $o_{max} = d_{max} - d_{min}/d_{max} + d_{min}$, where d = observed counts in a particular direction (Forward and Waterman, 1973)], the 19.5% response of stage II zoea is in fact one of the highest percentages published to date for a laboratory study. The best response obtained elsewhere was 20% for the copepod *Cyclops vernalis* (Umminger, 1968). Other values, which appear slightly lowered since they represent responses within 5° rather than 10° sectors, range from 16% for *Daphnia* (Jander and Waterman, 1960), to 8% for the ghost crab *Ocypode ceratophalma* (Daumer, Jander and Waterman, 1963), 6% for *Mysidium gracile* (Jander and Waterman, 1960), and even as low as 4% for the mite *Arrenurus* (Jander and Waterman, 1960). Measurement of behavior in the laboratory rather than in the natural conditions of the field may explain the rather low percentages of even the most significant of these responses. Concerning direction, the perpendicular and parallel swimming of *R. harrisi* in relation to the e-vector are typical polarotactic responses (see Waterman, 1974, for a summary of previous work).

It is important to consider whether the observed polarotaxis is an independent behavior from a phototaxis to light intensity distribution patterns created by the interaction of polarized light with the experimental vessel. The experiments with the collar of alternating white-dark quadrants surrounding the experimental vessel strongly suggests that the two behaviors are independent.

Comparison of the two responses under similar laboratory conditions (Forward, 1974; Forward and Costlow, 1974) provides further evidence for their independence. While the phototactic behavior itself and the spectral and intensity sensitivity for phototaxis were almost identical at all four zoeal stages, polarotaxis varied dramatically. Stages I and IV showed no responses. Even though stages II and III showed polarotaxis, the orientation direction is different, since stage II swims perpendicular to the e-vector while stage III swims parallel. The difference between the consistent phototaxis at all zoeal stages versus the variation in polarotaxis is a good indication that the two responses are actually different.

A difference in the optimal intensities for phototaxis and polarotaxis also suggests that they are separate responses. Polarotaxis is at a maximum at 10^{-2} W/m^2, which falls between the intensity that initiates maximum positive phototaxis by light-adapted animals (7 W/m^2, stage II; 10^{-1} W/m^2, stage III) and that for negative phototaxis ($10^{-2} - 10^{-4}$ W/m^2; Forward and Costlow, 1974). It may be significant that 10^{-2} W/m^2 is an intensity of transition for phototaxis at which not only are positive and negative phototaxes reduced, but random swimming increases (R. B. Forward, Jr., unpublished), while polarotaxis is maximal at this intensity.

Comparison of the spectral sensitivity curves for polarotaxis and phototaxis shows that both are maximal at 500 nm (Forward and Costlow, 1974). The response spectrum for phototaxis has another smaller maximum at 400 nm, and it appears from the rise seen at 420 nm in the sensitivity spectrum for polarotaxis (Fig. 7) that it may also have a secondary maximum in this region. Unfortunately, neither the tungsten light source nor the polaroid utilized in these experiments permitted the examination of wavelengths below 420 nm. While a dual pigment system is suggested by two maxima in the spectral sensitivity curve, further experiments utilizing selective wavelength bleaching and/or pigment extractions are necessary for positive verification. Nevertheless, the spectral maxima shown here for *R. harrisi* are consistent with those for other adult brachyuran crustaceans with dual pigment systems (Wald, 1968; Wasserman, 1974), as well as with those for animals which have only one pigment, *e.g., Callinectes sapidus,* 505 nm (Goldsmith and Fernandez, 1969) and *Carcinus maenus,* 508 nm (Horridge, 1967).

Although *R. harrisi* may possess a mutliple pigment system, the identity of the spectral characteristics of phototaxis and polarotaxis provides no evidence for a functional separation of pigments for these responses, as has been suggested for some terrestrial arthropods, *e.g.,* the ant *Cataglyphis bicolor* (Duelli and Wehner, 1973) and the bee *Apis mellifera* (Menzel and Snyder, 1974), in which polarization sensitivity is restricted to ultraviolet sensitive cells.

The suppression of polarotaxis in *R. harrisi* following dark adaptation is in marked contrast to the phototactic response, in which positive phototactic intensity sensitivity is increased by 1–2 log units in dark adapted animals (Forward, 1974; Forward and Costlow, 1974). One potentially viable explanation, based on a model of an insectan fused rhabdom, concerns electrical coupling between retinula cells (Snyder, Menzel and Laughlin, 1973). If cells with perpendicular microvilli were coupled in dark adaptation only, as Shaw (1969a) suggests is possible, then Snyder *et al.* (1973) assert that the resulting overall sensitivity increase would be at the expense of spectral and polarization sensitivity. While Muller (1973) suggests that only parallel tubules are coupled in the crustacean-type rhabdom, thereby increasing rather than eliminating polarization sensitivity, Mote (1974) argues against this interpretation. Although he refutes the theory of parallel coupling, it is still unclear whether the coupling of perpendicular tubules is possible given the interdigitating microvilli of the crustacean rhabdom. Although such a case could explain both the increased sensitivity in phototaxis and the elimination of polarotaxis with dark adaptation, our data offer no basis for an assertion of this mechanism in *R. harrisi.*

In interpreting these results, it must be remarked that due to a certain degree of artificiality, laboratory experimentation approaches the question of behavior in terms of *capability,* not *actuality.* While these experiments therefore do not result in a description of behavior in the field, our demonstration of larval polarotaxis does reveal that the larvae can perceive and orient to linearly polarized light. Only experimentation in the natural conditions of the field, however, can definitively answer questions of actual behavior, and although submarine patterns of polarization are well described (Waterman, 1954; Waterman and Westell, 1956), actual field experimentation on underwater polarotaxis has been minimal. The detailed studies which do exist concern fish (Waterman and Forward, 1970, 1972), and so except for a preliminary study by Bainbridge and Waterman (1957), the field behavior of aquatic arthropods including plankton and larval forms remains entirely unexamined.

However, results of field studies performed on the orientation of terrestrial arthropods (*e.g.,* von Frisch, 1967; Duelli and Wehner, 1973) strongly suggest a major role for polarization within the mechanism of sun compass orientation. For example, in the desert ant, *Cataglyphis bicolor,* Duelli and Wehner (1973) found solar location to be unimportant relative to sky polarization. This result confirms, for the terrestrial case at least, that polarization cues are used in the field.

The lack of comparable information for the underwater case has created a disjunction between laboratory and field behavior of aquatic arthropods which is critical to the creation of a general theory of the use of polarized light. This problem arises from the fact that there are basically three avenues of investigation of polarized light: perception, behavioral capability and normal field behavior.

Although studies within each of the areas can clearly yield useful and necessary information, their integration is precluded because the nature of the links between the area is unclear. Specifically, we neither know how the electrical signal from the retinula cells is analyzed and converted into behavior, nor if polarized light underwater is even used by animals in nature. Laboratory studies of orientation behavior are therefore separated from both polarized light reception and its use in nature by the lack of knowledge about central nervous system information processing and behavior in the field.

By preventing the formulation of a unified theory, these gaps reduce the utility of information within the three areas of investigation. We therefore feel that investigations into the areas of information processing and field behavior are critical at this stage in the study of polarized light and navigation by aquatic arthropods.

This work was supported by Environmental Protection Agency Grant No. R801128–01–1. We thank Professors John D. Costlow, Jr., and C. G. Bookhout for many helpful comments and the use of their culturing facilities during this study, Mrs. Karen Johnson and Mr. John Freeman for general technical assistance and Professor G. A. Horridge for his comments on the manuscript.

Summary

1. To determine the ontogeny plus the intensity and spectral characteristics of polarotaxis in larvae of the crab, *Rhithropanopeus harrisi,* swimming paths in relation to the e-vector direction of linearly polarized light were monitored and recorded with a closed circuit video system.

2. All four zoeal stages were examined. Only stages II and III responded with significant directional swimming (stage II, perpendicular to the e-vector, stage III, parallel to the e-vector; Figs. 3 and 4). Stages I and IV appeared indifferent to the polarization plane (Figs. 2 and 5). Swimming under unpolarized light was consistently random (Table I).

3. Polarotaxis disappeared following dark adaptation of stage II larvae (Fig. 6).

4. The lower intensity threshold at 499-nm light for polarotaxis was found to be between 10^{-4} and 10^{-3} W/m^2, while the upper threshold is in the range 10^{-1} to 2×10^{-1} W/m^2 (Figs. 3 and 4).

5. Examination of selected wavelengths from 420–620 nm yielded greatest responsiveness at 499 nm (Fig. 7). A rise evident at 420 nm suggests a secondary maximum in the violet.

6. Stage II larvae showed significant positive phototaxis under depolarized light when a collar of alternating black and white quadrants surrounded the experimental vessel. With the e-vector of polarized light aligned parallel to the white sectors, phototaxis was reduced by polarotactic swimming into the black sectors (approximately perpendicular to the e-vector).

7. A comparison with phototaxis (Forward and Costlow, 1974) provides two items of evidence that polarotaxis and phototaxis are indeed separate responses.

First, phototaxis is essentially unchanged during larval development, while polarotaxis appears only at stages II and III. Secondly, the responses have different optimum intensities.

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CONTENTS

Annual Report of the Marine Biological Laboratory 1

ANDERSON, PETER A. V. AND JAMES F. CASE
Electrical activity associated with luminescence and other colonial behavior in the pennatulid *Renilla kollikeri* 80

BEITINGER, THOMAS L.
Diel activity rhythms and thermoregulatory behavior of bluegill in response to unnatural photoperiods 96

BLAKE, JAMES A. AND KEITH H. WOODWICK
Reproduction and larval development of *Pseudopolydora paucibranchiata* (Okuda) and *Pseudopolydora kempi* (Southern) (Polychaeta: Spionidae) 109

BROWN, JUDITH A.
Sidereal-day variation in spontaneous activity of the mouse, *Mus musculus* 128

BROWN, STEPHEN C.
Biomechanics of water-pumping by *Chaetopterus variopedatus* Renier. Skeletomusculature and kinematics 136

CHIPPENDALE, G. M. AND C.-M. YIN
Reappraisal of proctodone involvement in the hormonal regulation of larval diapause 151

FINGERMAN, MILTON, SUE W. FINGERMAN AND DREW T. LAMBERT
Colchicine, cytochalasin B, and pigment movements in ovarian and integumentary erythrophores of the prawn, *Palaemonetes vulgaris* . 165

LYERLA, TIMOTHY A., JOANN H. LYERLA AND MARILYN FISHER
Pigmentation in the orange tunicate, *Ecteinascidia turbinata* 178

MAZURKIEWICZ, MICHAEL
Larval development and habits of *Laeonereis culveri* (Webster) (Polychaeta: Nereidae) 186

MITCHELL, K. A.
An analysis of shell occupation by two sympatric species of hermit crab. I. Ecological factors 205

NIJHOUT, H. FREDERIK
A threshold size for metamorphosis in the tobacco hornworm, *Manduca sexta* (L.) 214

RAVINDRANATH, M. H.
Effects of hydrogen ion concentration on the morphology of hemocytes of the mole-crab *Emerita asiatica* 226

SPENCER, ANDREW N.
Behavior and electrical activity in the hydrozoan *Proboscidactyla flavicirrata* (Brandt). II. The medusa 236

VIA, SARA E., AND RICHARD B. FORWARD, JR.
The ontogeny and spectral sensitivity of polarotaxis in larvae of the crab *Rhithropanopeus harrisi* (Gould) 251

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Continued on Cover Three

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SKIN IMPULSES AND LOCOMOTION IN *OIKOPLEURA* (TUNICATA: LARVACEA)

Reference: *Biol. Bull.*, **149**: 267–286. (October, 1975)

Q. BONE AND G. O. MACKIE

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Larvaceans are small pelagic tunicates which live in gelatinous houses that they secrete, drawing a feeding current of water through the house by oscillating the tail. Their anatomy and histology have been studied by a number of workers (*e.g.* Fol, 1872; Seeliger, 1893; Lohmann, 1933; and Martini, 1909). Although special methods for nerves were not used by these workers, the living animals are very transparent, so that it is possible to see some sensory structure in life, as well as the motor innervation of the caudal muscle cells. The organization of the nervous and muscular systems was therefore fairly accurately described long ago; it is seen diagrammatically in Figures 1 and 2.

An anterior "brain" containing a statocyst lies dorsally in the trunk region, from it nerves run to the lips and to the pair of ciliated stigmata through which the feeding current passes (Galt and Mackie, 1971). A dorsal nerve cord running over the stomach joins a caudal ganglion near the base of the tail; from the caudal ganglion a caudal nerve passes down the tail beside the notochord. During development, the tail rotates to lie in the horizonal plane, but the caudal nerve is morphologically dorsal to the notochord, as seen in Figure 2.

In the nerve cord of the tail there are conspicuous nerve cell bodies (Fig. 2), often paired and segmental in arrangement, reflecting the segmental origin of the motor axons, which terminate upon the inner surface of the caudal muscle cells in beautiful corymbiform motor endplates.

Other finer fibers (figured by Fol, 1872) also pass segmentally out of the nerve cord and terminate in branching, sometimes beaded, endings on the inner surface of the muscle fibers. Martini (1909) suggested that they were sensory terminations; it is, however, probable that these axons too are motor.

A pair of nerves (the *nervi recurrentes caudae* of Langerhans) pass forward from the caudal ganglion across the dorsal and ventral haemocoel of the tail of the

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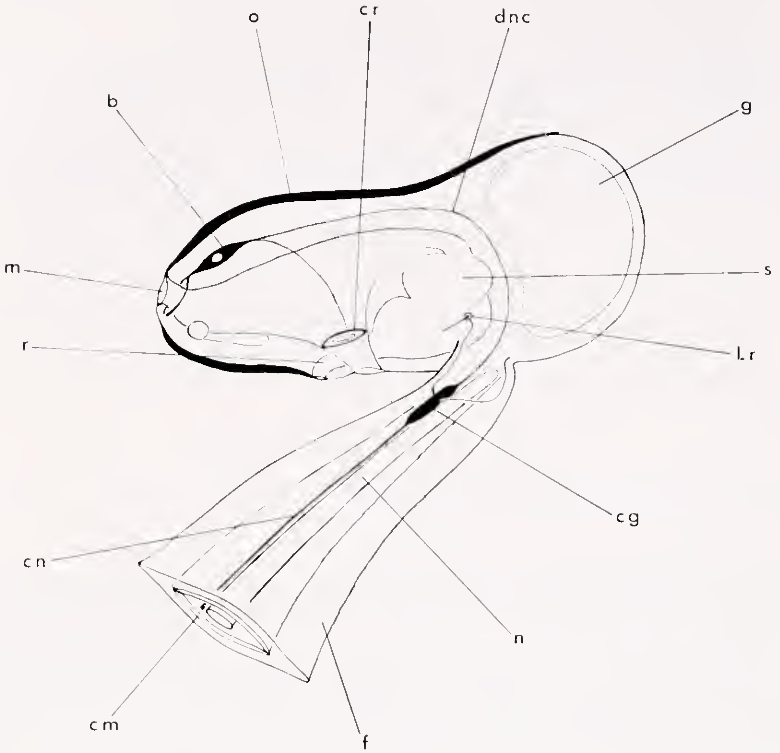

FIGURE 1. Diagram showing organization of trunk and anterior tail region in *Oikopleura*. The tail has been twisted for clarity. b represents brain; cg, caudal ganglion; cn, caudal nerve; cm, caudal muscle cells; cr, ciliary ring; dnc, dorsal nerve cord; f, fin; g, gonad; Lr, Langerhans receptor; m, mouth; n, notochord; o, oikoplastic epithelium; r, rectum; and s, stomach.

paired bristle-bearing receptor cells on either side of the trunk which were discovered by Langerhans (1877). Seeliger (1893) suggested that these cells were sensitive to vibration. Apart from the receptor cells of the lips, the Langerhans receptor is the only exteroreceptor which has been observed in *Oikopleura*.

The muscular system operating the tail is composed of a small number of muscle cells (8–12 depending upon the species) lying on either side of the notochord and extending caudally beyond the notochord tip. These cells interdigitate with adjacent cells in a characteristic way (Bogoraze and Tuzet, 1969) and have an inner myofibrillar zone and an outer mitochondrial zone. They are focally innervated by the corymbiform endplates, and transmission is apparently cholinergic (Flood, 1973).

The outer surface of the animal is not covered by a tunic, as in other tunicates, but is composed of an exposed epithelial layer consisting of thin polygonal cells; in the oikoplastic regions of the trunk the cells are thicker and highly specialized for secretion of the house.

Although the structure of *Oikopleura*, summarized briefly above, is fairly well known, little is known of the physiology of the animal. In a previous paper (Galt and Mackie, 1971) the electrical correlates of ciliary reversal were investigated. It was also found that small potentials could be recorded from the surface of the trunk when the epithelium was touched; these events were suggested to be propagated skin impulses.

In the present paper we show that the epithelium covering the animal is indeed capable of propagating skin impulses, and that these are linked to the neurons driving the locomotor rhythms of the animal.

MATERIAL AND METHODS

Two species of *Oikopleura* were invesigated. Unless otherwise stated, the species referred to in this paper is the large *Oikopleura labradoriensis* Lohmann, which was common in the plankton off the Friday Harbor laboratory throughout April 1974. Large specimens of this species had tails some 7 mm long. By June it had become scarce, and the smaller *O. dioica* Fol was used for some experiments. The animals were dipped from the water off the laboratory dock, and transferred

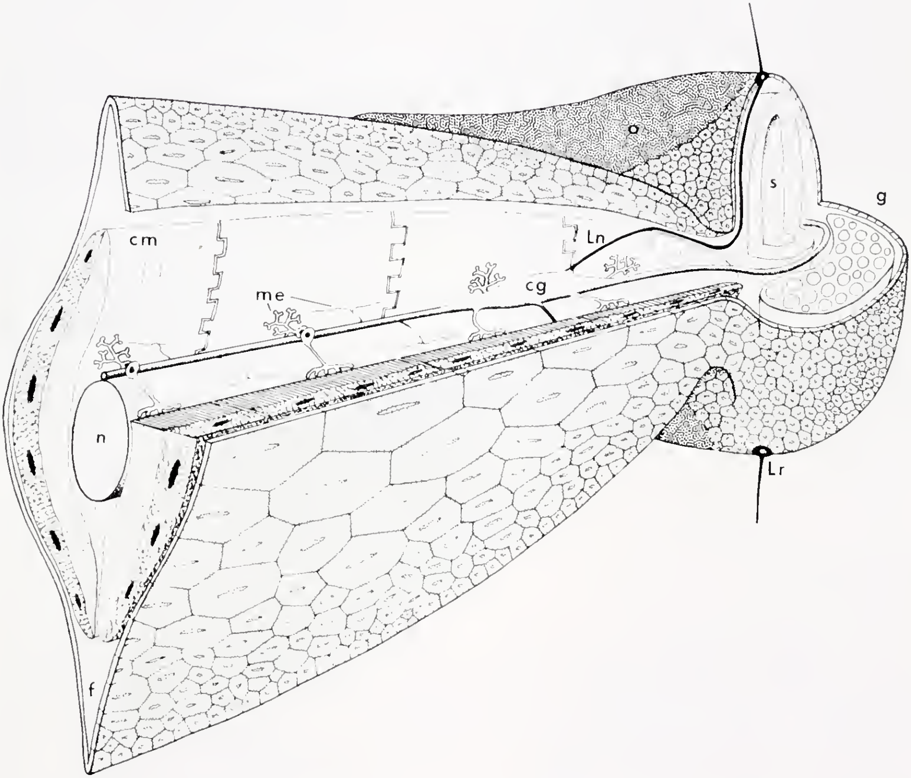

FIGURE 2. Stereogram of anterior caudal region. The oikoplastic epithelium of the trunk region is shaded. Note the Langerhans nerves arising from the caudal ganglion and passing to bristle-bearing receptor cells, and two types of motor ending. cg represents caudal ganglion; cm, caudal muscle cell; f, fin; g, gonad; Ln, Langerhans nerve; Lr, Langerhans receptor cell; me, motor endings; n, notochord; and o, oikoplastic epithelium.

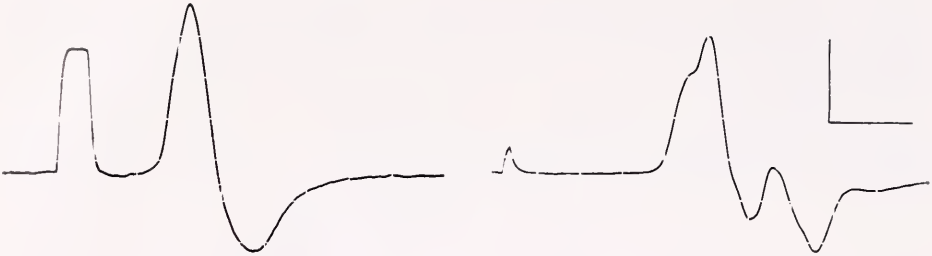

FIGURE 3. Skin impulses evoked by electrical stimulation of isolated fin region; scale bars: 500 μv, 4 msec.

to small aquaria in the laboratory for observation of their behavior while still in their houses.

Electrical recordings were made from specimens held by suction electrodes or pinned down on Sylgard platforms with cactus spines. The recording dish was mounted on a Peltier cooling ring.

Plastic suction electrodes were used for external recording. Sea water was drawn up into the tube, and the animal was held to the tip by suction. Such electrodes were also used for stimulation. Input was fed to a Grass 79C polygraph with EEG amplifiers, and the amplified signal was displayed concurrently on a Tektronix 5103N storage oscilloscope. Stimulating shocks of 1–2 milliseconds duration were given with a near-threshold voltage setting.

In some experiments, input from the suction electrodes was fed to a Gould Brush 220 oscillograph, *via* the amplifiers of a Tektronix 502 oscilloscope.

Living and Flemming-fixed specimens were examined by Nomarski interference microscopy; material for electron microscopy was fixed in 2.5% glutaraldehyde in Millonig's phosphate buffer, followed by postfixation in 1% osmium tetroxide. Sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300.

RESULTS

Skin impulses

When suction electrodes are attached to any part of the surface of the tail or posterior trunk region, and the skin is stimulated electrically or mechanically, it is possible to record impulses which spread across the surface from the point stimulated. These are large events, (1–2 millivolts), with an initial positive going component; the form of the pulse being rather variable (Fig. 3a and b). By placing a stimulating electrode on the tail tip, and two recording electrodes at different distances away from it, records such as Figure 4 are obtained. Within the areas in which they are conducted, the skin impluses show non-decremental all-or-none spread. Conduction velocities lie within the range 15–21 cm/sec at 12–13° C, with an average of 17.8 cm/sec for a number of records from different specimens.

These values refer to the first impulses obtained from preparations which had not been stimulated in the recent past. Conduction velocity decreases with repeated stimulation, the rate of decline being related to frequency of stimulation as shown in Figure 5. Measurements from preparations where the impulse passes successively beneath two recording electrodes show that the increase in conduction time represents a decrease in conduction velocity, not an increased initiation lag.

The impulses spread diffusely in all directions. They can pass around the edge of the tail fin and invade the contralateral side. They spread to (and can be evoked from) the epithelium covering the gonad and hinder trunk region, but are not found in the specialized oikoplastic epithelium of the anterior trunk region (Figures 1 and 2). Stimulation in this region does not yield a behavioral response except near the lips, where sensory cells are located. Lip stimulation affects the ciliary beat of the stigmata, the response being mediated by a nervous pathway (Galt and Mackie, 1971).

We have not succeeded in making intracellular records from the single layer of epithelial cells some 3 μm thick, which forms the skin of the animal, but there is compelling evidence that the skin impulses are in fact conducted by these cells.

The impulses spread diffusely across the surface of the animal, and in the regions where they spread, there is a uniform degree of excitability, so far as we can determine. Interruption of the epithelial sheet by abrading the skin around the tail blocks conduction across the damaged belt. On the other hand, deep incisions into the tail, including cuts which sever the nerve cord, do not block conduction provided that a skin bridge is left intact. The impulses can be recorded

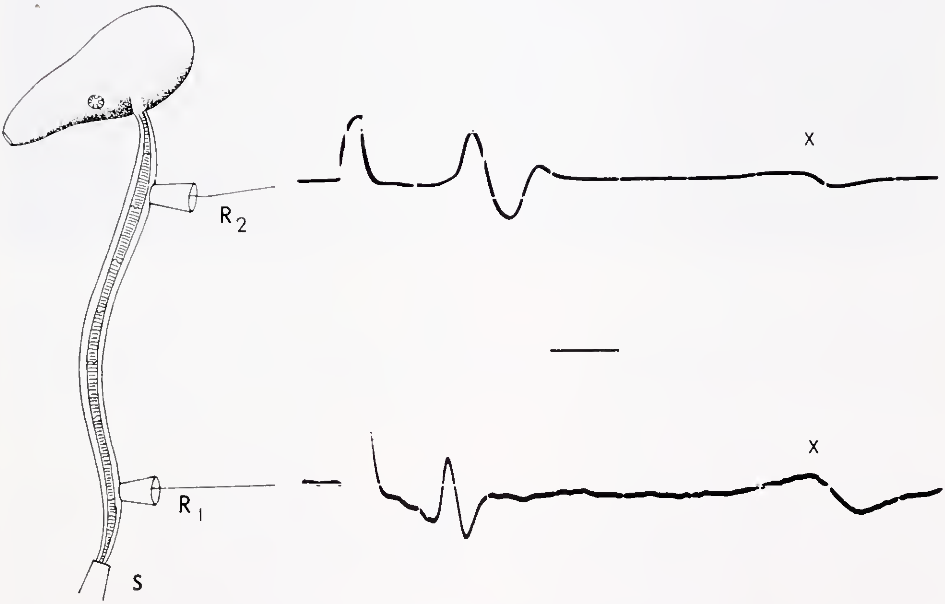

FIGURE 4. Propagation of skin impulses. The position of stimulating (S) and recording electrodes (R_1, R_2) are shown at left. The cross marks the beginning of tail movement evoked by the skin impulse.

from isolated strips of the tail fin, which consists essentially of a simple outfolding of the skin. Nerve fibers in the tail can easily be followed in the living animal to their terminations associated with the inner face of the muscle bands.

If there was a general innervation of the skin, or a nerve net underlying it, this should be readily visible, but no such elements have been seen, either in the living animal, or in numerous EM sections of the skin in the two species examined. The only nerves which reach the skin in the areas we are concerned with are those which go to the two lateral bristles on the posterior ventral trunk wall, which, as we show below, provide the pathway whereby skin impulses reach the central nervous system. Fol (1872) described (in another species) a caudal receptor cell and fiber lying at the extreme tip of the tail; in the two species we have examined, a cell with a long process is found at the tip of the tail, but it does not seem to be in connection with the nerve cord, and is probably a connective tissue cell.

The epithelial cells of the skin are connected by gap junctions (see below) which offer a potentiality for electrical, ionic, and metabolic communication (Gilula, Reeves and Steinbach, 1972). Conduction across an electrically-coupled sheet of cells would not be suppressed by drugs which block cholinergic synapses. We find that skin conduction continues in preparations treated with levels of Mg^{++} which suppress muscle rhythms, presumably by blocking the nervous pathways through which the muscle is activated.

Skin impulses having many features in common with those described above have been demonstrated in hydrozoa and in amphibian larvae. In the latter, intracellular records have confirmed that the events originate within epithelial cells. We have also found that skin impulses are present in the tadpole larvae of the ascidian *Dendrodoa grossularia,* and have been able to record these intracellularly from the epithelial cells of the tail (Mackie and Bone, in preparation). Finally, skin impulses are not found in the larvacean *Fritillaria pellucida,* and histological examination shows that epithelial cells are absent in this species (Bone, Fenaux and Mackie, in preparation).

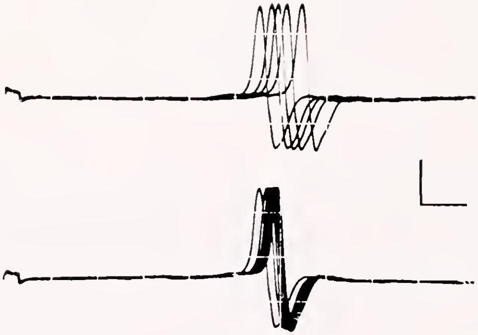

FIGURE 5. Decline in conduction velocity of successive skin impulses evoked by electrical stimulation. The upper is stimulated at 0.5/sec; the lower is stimulated at 1.5/sec; scale bars: 500 μv; 5 msec.

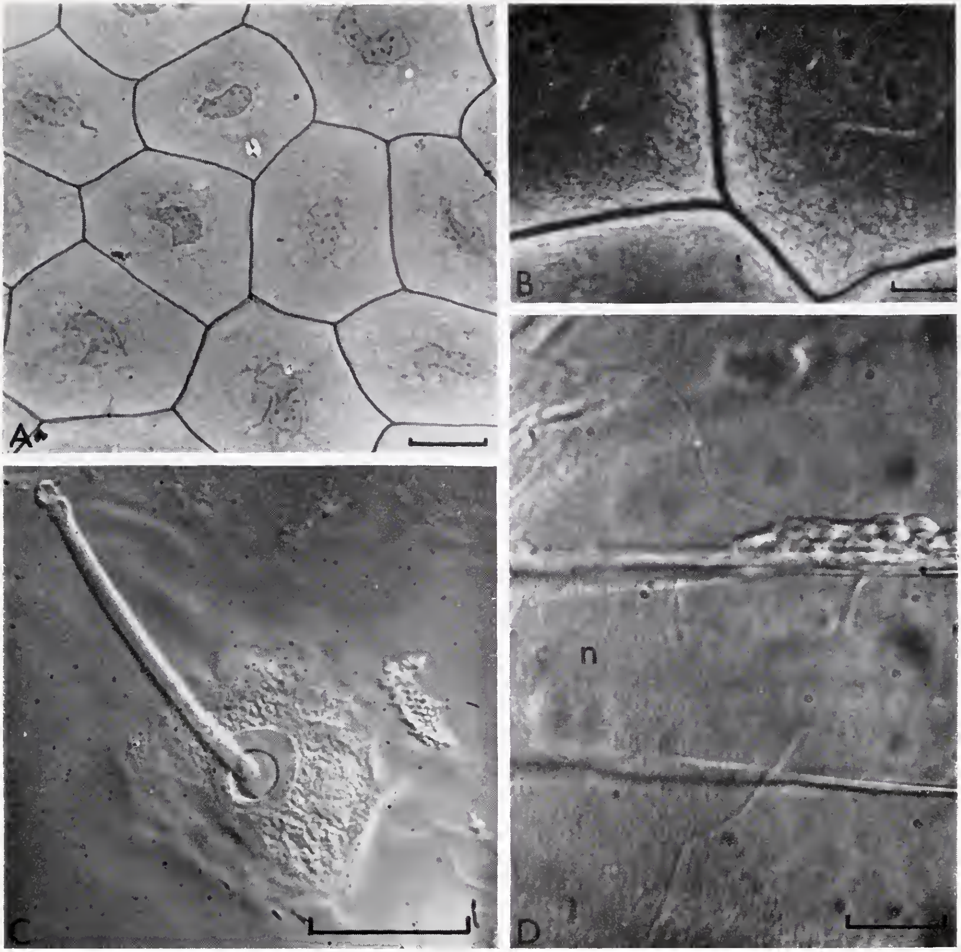

FIGURE 6. a) Epithelial cells covering gonad region fixed in Flemming without acetic and stained with iron haematoxylin; b) the same region as (a) at a higher magnification showing the fibrous appearance of the cytoplasm; c) the Langerhans receptor cell; in this preparation, which was fixed in Flemming without acetic and examined with Nomarski interference contrast, the tip of the bristle has been damaged; d) The caudal ganglion and Langerhans nerves seen in the living animal with Nomarski intereference contrast. The junction of the tail with the trunk is to the left of the figure; n indicates the notochord.

These considerations leave little doubt that the skin impulses of *Oikopleura* are propagated across the epithelial cell sheet which forms the outer surface of the animal. The wave forms recorded with suction electrodes show considerable variation. In their simplest form, skin impulses are smooth biphasic events, but secondary blips, shoulders and composite wave forms are also seen. It must be borne in mind that suction electrodes vary in bore and resistance; in the amount of tissue they cover or imbibe (partly dependent upon the suction applied); in the extent to which they damage the cells under or around the point of attachment;

and the extent to which they may leak under the stress of sudden movement. Some distortion of what is assumed to be a conventional waveform is inevitably to be expected in recordings made with this technique. Distortion resulting from capacity-coupling in the amplifiers is probably slight, since records made with d-c amplifiers differ little from those (figured in this paper) using a-c coupled amplifiers.

In our records, the typical skin impulse has a duration of 8–12 msec. There is a correspondingly short refractory period of some 5 msec.

Little or no attenuation of the second impulse occurs with pulse pairs 12 msec apart, again suggesting that the membrane is fully repolarized within that period.

Impulses propagated in the skin pass to the locomotor centers in the central nervous system and either evoke a swimming burst, or accelerate existing swimming activity, as we discuss in a separate section below. In fresh specimens there is a one-to-one coupling between skin pulses and initial muscle response. There are no cilia on the outside of the animal nor secretory cells in the conducting regions, so that in the absence of such local skin effectors, there is no reason to suppose that skin impulses act other than as part of the afferent sensory pathway in the locomotory escape response.

Structure of the epithelial cells of the skin

The cells which propagate the skin impulses are large flattened polygonal cells (Fig. 6, a and b) whose borders interdigitate slightly.

Larger cells cover the lateral surface of the tail than elsewhere (Fig. 2). The largest are around 250 μm across, elongated in the anterioposterior direction, as are their spindle shaped nuclei. The ultrastructure of these cells is striking, for they are divided into two distinct layers; an outer fibrous or "cuticular" layer without mitochondria or other cell organelles, and a thin inner layer containing the nucleus, mitochondria, Golgi appartus, and rough ER (Fig. 7). It is possible that the fibrous layer of the epithelial cells is made up of tunicin fibrils, since in size and arrangement, the fibers resemble those in the test of other tunicate groups. On the outer surface of the cell is an irregularly beaded layer. This peculiar arrangement suggests that the epithelial cells are divided functionally into an outer "cuticular" zone, and an inner zone in which the vital functions of the cell take place. As we shall see in a later section, *Oikopleura* is remarkably impermeable to drugs if the epithelial layer is intact. Unlike other tunicates, the adult *Oikopleura* does not possess a tunic, for although present in the larva, the tunic ruptures and is lost as the animal increases in size (Galt, 1972). This unusual outer layer of the epithelial cells is not present in the ascidian tadpole larva (Mackie and Bone, in preparation), which is equally impermeable to drugs, but which *does* possess a tunic. Adjacent epithelial cell membranes are separated by a gap of some 200 Å (Fig. 7), except for a limited region near the bases of the cells where the two membranes are more closely apposed forming gap junctions. Since these gap junctions are not visible in every section of two adjacent cells, it appears that they do not form a continuous band around the bases of the cells, rather that they are distributed in a punctate manner.

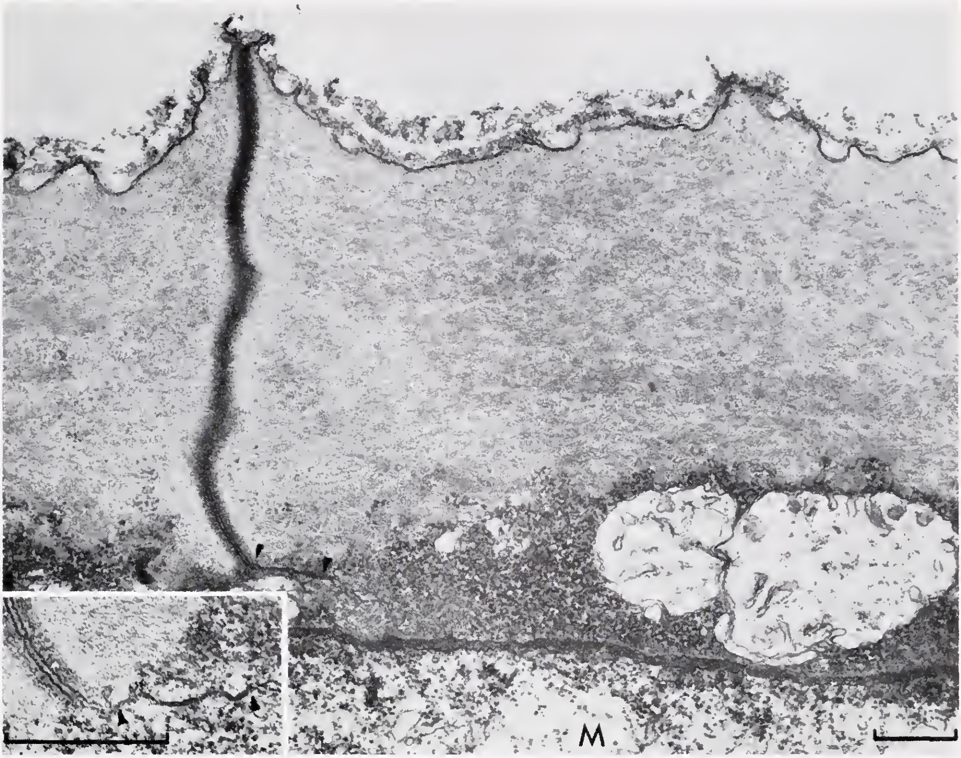

FIGURE 7. Junction between two epithelial cells of trunk. Note outer "cuticular" region of epithelial cell, and inner mitochondrial zone. The adjacent cell membranes lie 200 Å apart (inset) except for a gap junction (arrowed) near the inner face of the cells. M represents underlying muscle cell; scale bar 0.5 μm.

Motor patterns of the tail

The activity of the caudal musculature in *Oikopleura* (studied in *O. dioica* by Galt, 1972) is essentially rhythmic, varying from long cycles of oscillation to single beats at intervals; usually short bursts of tail movement alternate in a regular way with periods of inactivity (Fig. 8). Both amplitude and frequency of the tail movements are variable, so also are the lengths of the bursts of activity and the interburst periods. Our records of these patterns of activity were obtained by attaching suction electrodes to the trunk or tail; potentials serving to indicate tail movements arise as the animal moves its tail, but it is not certain whether these are movement artifacts or whether at least in part, they represent electrical activity of caudal muscle cells, as Galt and Mackie (1971) previously suggested.

Glass microelectrodes placed with their tips in the caudal muscle cells of animals whose tail movements have been restricted by pinning down the tail, provide rather similar records (compare Fig. 8 a and b), but similar difficulties of interpretation arise. It seems on the whole most probable that the potentials recorded by suction electrodes are extracellularly recorded muscle potentials.

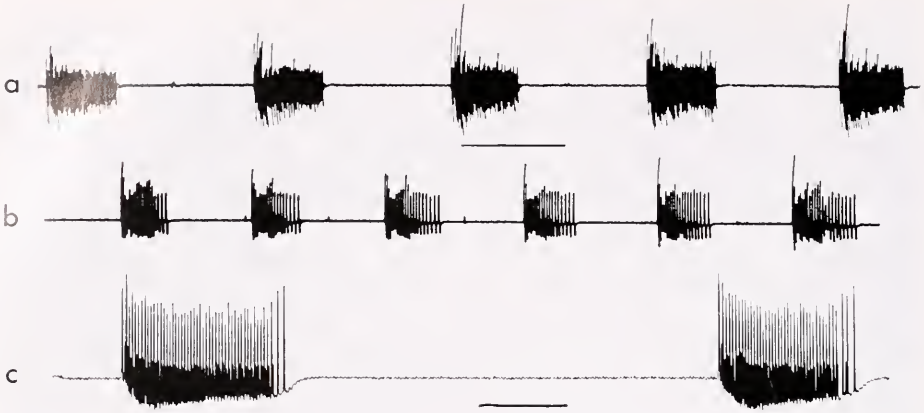

FIGURE 8. Swimming activity: (a) suction electrode record; (b) record obtained from microelectrode (in caudal muscle cell); (c) suction electrode record (*O. dioica*); scale bars: a and b: 10 sec; c: 1 sec.

Three general patterns of spontaneous activity can be distinguished in intact animals: pumping behavior in the house; swimming behavior; and house rudiment expansion behavior.

Pumping behavior. When occupying the house in which it feeds, *Oikopleura* pumps water through the house (thus moving the house forward through the

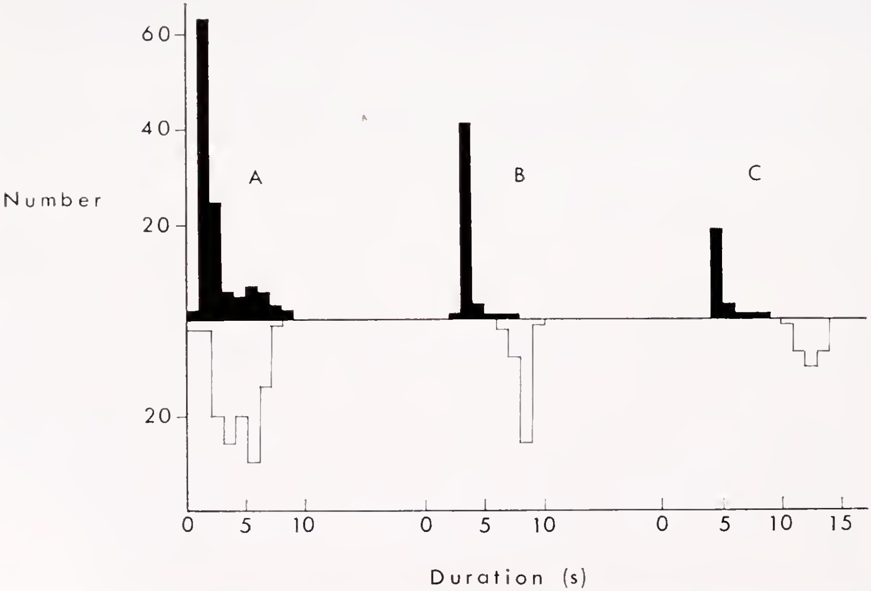

FIGURE 9. Length of burst (filled) and interburst (open) periods during periods of: (a) pumping behavior in the house; (b) swimming behavior of intact animal attached to electrodes; and (c) isolated tail attached to electrodes.

water) by rhythmic bursts of oscillation of the tail at frequencies between 2.5 and 3.0/sec. This activity is cyclical; in an animal observed for 25 min in an aquarium, the length of the bursts of tail activity varied somewhat, as did the interburst intervals, but over short periods, both are very regular (Fig. 9). These low frequency bursts characteristic of pumping behavior within the house, may be shown for a few minutes after animals have been removed from the house and attached to suction electrodes, but more usually, although burst length and interburst interval remain similar, the frequency of oscillation within bursts increases almost at once, and a different pattern of activity appears.

Swimming behavior. When feeding in the house, *Oikopleura* is very sensitive to vibration, even light touch of the house causing the animal to leave in a burst of swimming at relatively high frequency. This is then followed by a similar rhythm of activity to that of pumping behavior, but at higher frequency, usually between 5 and 6 c/sec within the bursts of activity. The animal swims upwards in the water column, and sinks vertically head downwards during the periods of inactivity. The timing of bursts and interburst periods in animals swimming freely in aquaria is similar to that shown when they are attached to suction electrodes; for a given animal over periods of 15–20 min or so, this pattern of activity is remarkably regular (Fig. 9b). Over longer periods, the pattern may change; it is slightly different between different animals. During the swimming burst, the frequency of tail oscillation usually remains the same (see Fig. 12).

Removal of the anterior part of the trunk (containing the cerebral ganglion), or separation of the tail from the trunk by cutting across the base of the tail, does not significantly alter the swimming pattern; the isolated tail (Fig. 9c) shows a similar pattern to the intact animal. Successive removal of portions of an isolated tail beginning at the tail tip does not abolish the swimming rhythm even if only 2 muscle cells remain on either side of the tail stub, but if the tail is progressively shortened in a similar way, beginning at the base, then spontaneous activity ceases as soon as that portion of the tail containing the caudal ganglion has been removed. Isolated tails which have been cut across so as to remove the caudal ganglion not only do not exhibit spontaneous activity, but respond to high levels of electrical stimulation with single beats only, and rhythmic activity cannot be

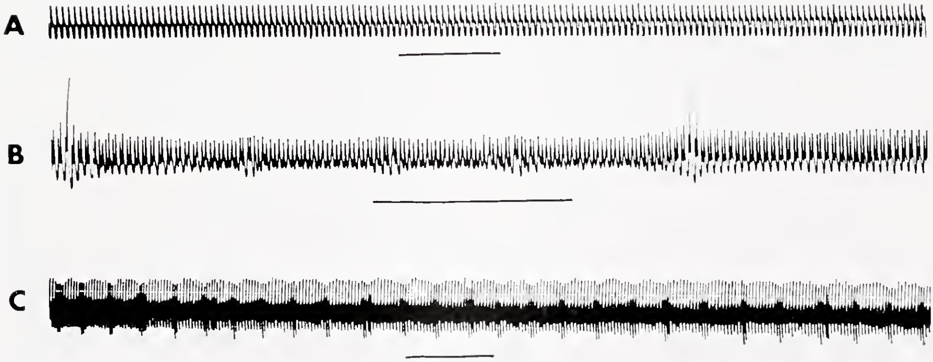

FIGURE 10. House rudiment expansion behavior. Note larger rhythm superimposed on steady oscillation in B and C; scale bars: A, 10 sec; B, 5 sec; C, 15 sec.

evoked. Stimulation must be strong to excite such preparations, the muscle cells are presumably stimulated directly.

We conclude therefore that swimming behavior is dependent upon the presence of the caudal ganglion, and that input from the cerebral ganglion is not required for rhythmic swimming. This conclusion is supported by experiments in which the caudal ganglion is hemisected by transecting the tail at the level of the ganglion, or is interfered with mechanically by touching with a microelectrode. Experiments of this kind yielded preparations which either did not show any spontaneous activity or which showed single tail beats at 5 sec or 10 sec intervals. In one case, irregular tail beats at intervals of 1–12 sec appeared, with occasional bursts of up to 5–6 beats.

Such patterns of activity after partial destruction of the caudal ganglion are also produced by the action of various blocking agents upon the intact animal. *Oikopleura* is remarkably resistant to drugs such as curare, procaine and atropine, and also to high concentrations (up to 100 mM/lr) of Mg^{++}, Mn^{++} and Co^{++}. For example, animals will swim for 30 min or longer in equal parts of sea water and isotonic magnesium chloride solution. Evidently, the external epithelium is extremely impermeable (as its structure would suggest), and the situation is similar to that found for the ascidian heart by Kriebel (1968), where drugs such as acetylcholine and adrenalin are ineffective unless allowed access to the *inner* wall of the heart. When drug access to the muscles and nervous system of *Oikopleura* is allowed by cuts in the epithelial wall, curare and Mg^{++} reversibly block rhythmic activity, and procaine does so irreversibly. Before activity ceases, single tail beats at 5 sec or 10 sec intervals are produced by all three drugs.

Lower concentrations of curare (below 10^{-5}) sometimes induce a rhythm of single beats at 2 sec intervals, without abolishing bursts of swimming activity.

Our experiments were usually carried out between 12 and 13° C; in four experiments the effects of varying the temperature of the bath in which the animals lay were investigated. Swimming activity is not abolished by lowering the temperature until the bath freezes: above 17° C however, the animals begin to swim erratically and soon die.

Change in temperature between 4° C and 16° C does not significantly alter the frequency of oscillation of the tail during bursts of activity although heart rate, and the metachronal rhythm of the ciliary rings declines as the temperature falls. Interestingly enough, the frequency of ciliary reversal pulses (Galt and Mackie, 1971) is more or less linearly related to temperature.

House rudiment expansion behavior. This pattern of long bursts (up to 4 min) of tail oscillation at frequencies around 4–5/sec is shown by animals which are expanding the house rudiment secreted by the oikoplasts of the trunk (Fig. 10). It was first described in *O. dioica* by Galt (1972), who observed that in animals free to move, house rudiment expansion behavior (REB) resulted in a "nodding" of the trunk which presumably serves to free the rudiment from the underlying oikoplasts, and enables it to be expanded. If the house rudiment is removed from an animal showing the almost continuous activity of REB, or if the animal itself shakes off the rudiment, REB stops and swimming behavior begins. REB is not shown by isolated tails, nor by animals in which the lips have been cut away (damaging the oikoplasts as well as resulting in removal of

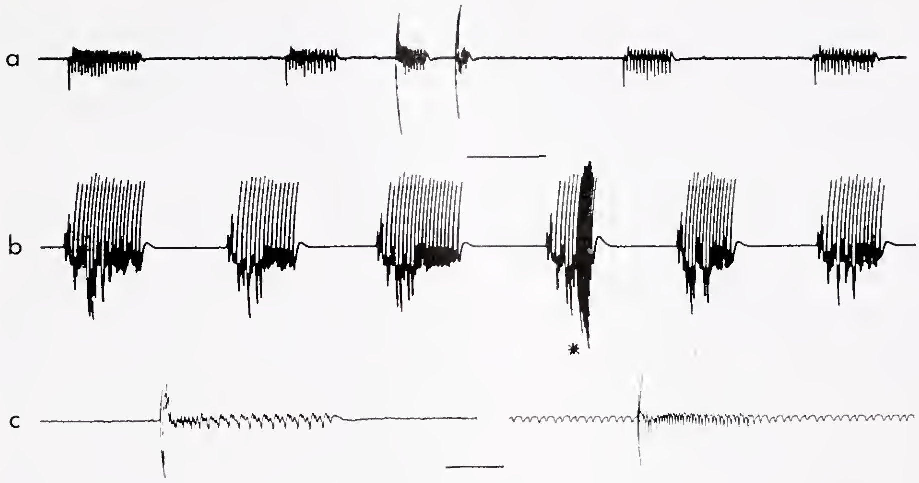

FIGURE 11. a) Skin impulses evoked by electrical stimuli reset the locomotor rhythm. Note that after the second skin impulse (the large potentials), a short swimming burst is evoked, and the interburst period after this is similar to that before the skin pulses; b) a skin impulse evoked by electrical stimulation (at the asterisk) accelerates the locomotor rhythm; c) two skin impulses evoked by electrical stimulation of the same animal. During an interburst period, the skin impulse elicits a burst of swimming, whereas during a period of steady oscillatory activity, the skin impulse accelerates the existing rhythm. The scale bars for the upper pair of records: 5 sec; for the two isolated skin impulses below: 1 sec.

the cerebral ganglion). It seems that this pattern of behavior is dependent upon input to the caudal ganglion from the cerebral ganglion.

Superimposed upon the 4–5/sec oscillations during REB are often larger rhythms marked by increased amplitude oscillations with little change in frequency (Fig. 10, B and C).

These are usually at intervals of 5–10 sec and may appear as gradual amplitude increases during the usual pattern of oscillation of REB, or may be single beats only of larger amplitude. It is striking that the larger rhythms superimposed upon the usual REB rhythm are of similar frequency to the rhythms observed when the caudal ganglion is partially destroyed, or when drugs are applied to the animal.

Resetting the motor rhythms. As noted above, skin impulses initiate movement in quiescent animals or increase the frequency of movement in animals which are swimming or expanding a house (Fig. 11). When animals are swimming in regular bursts, the interval between such an induced burst of activity and the succeeding spontaneous burst is the same as that between spontaneous bursts (Figs. 11a, 13a) so that the effect of stimulation is to reset the rhythmic pattern of activity, whether or not the induced burst of activity is shorter than preceding or succeeding bursts. The initial frequency of tail movement following stimulation is invariably much higher than that in spontaneous bursts, and declines during the burst in a linear manner (Fig. 12). Stimulation during REB may either result in a frequency increase, followed by a decline to a frequency below the characteristic 4–5/sec before the original frequency of oscillation is regained, or, rarely

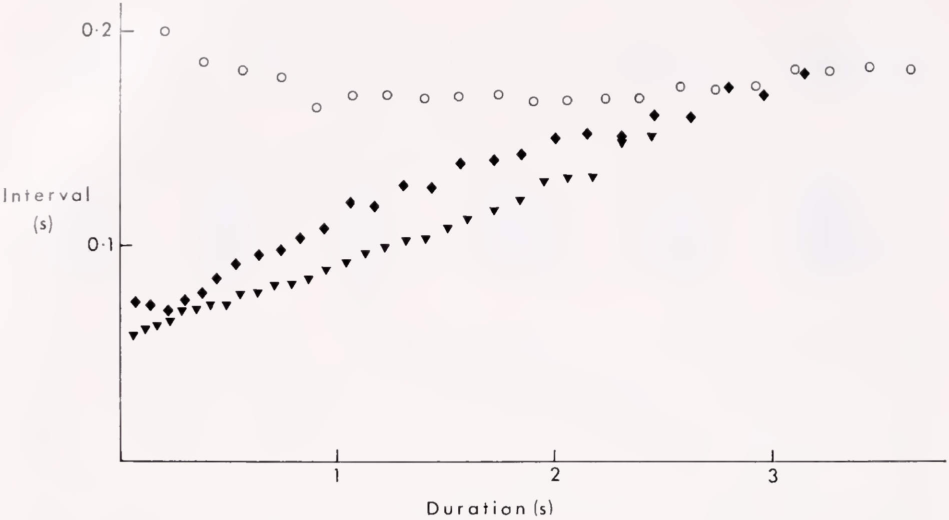

FIGURE 12. The frequency of swimming beat in a spontaneous swimming burst (open circles) compared with that in two bursts evoked by skin impulses (filled diamonds and triangles). The ordinate is the interval between successive tail movements; abscissa, the duration of the burst.

there may be no frequency increase, and the burst of REB activity stops shortly after the stimulus.

The afferent pathway from the skin. Since skin pulses evoke movements or accelerate movements in intact animals, and in animals which have had the anterior

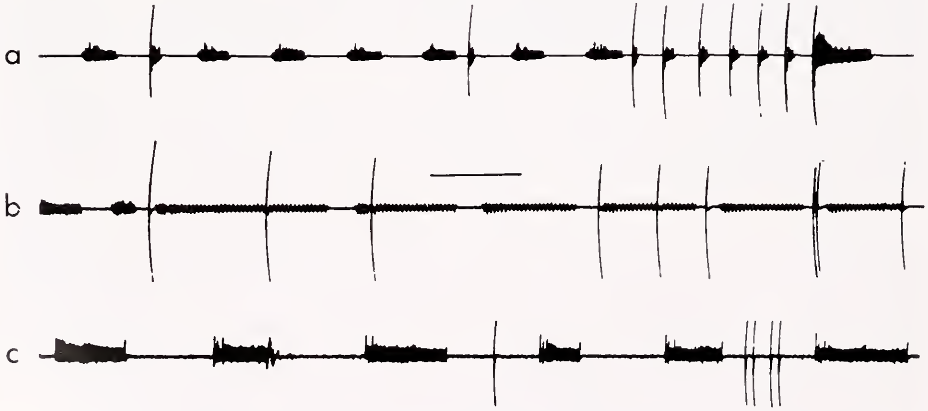

FIGURE 13. a) In the intact animal, skin impulses (large potentials) reset the locomotor rhythm; b) in the isolated tail, skin impulses do not affect the locomotor rhythm; c) after bi-lateral section of the Langerhans axons in an otherwise intact animal, skin impulses no longer reset the locomotor rhythm. In this record, the upper line indicates the stimulus and the large potentials on the lower line are skin potentials. For all records, the scale bar is 10 sec.

part of the trunk removed, but do not do so in isolated tails (Fig. 13b), it is evident that the link between the skin pulse and the central nervous system must lie in the posterior region of the trunk. An obvious route linking the skin with the caudal ganglion is provided by the paired bristle bearing cells lying on either side of the posterior trunk region, which are connected with the caudal ganglion by the nerves of Langerhans (Langerhans, 1877). Fortunately these nerves (actually single axons, 5 microns in diameter) are visible in the intact animal (Fig. 6d), so that it is possible to transect them to determine the effects of this operation on the transmission of stimuli to the cns. When recording electrodes are placed on mid-tail and on the trunk region, and a stimulating electrode is placed on the tail, it is found that unilateral section of the Langerhans axon does not block conduction between the skin and the caudal ganglion, whereas after bilateral section, although skin pulses pass from tail to trunk, they no longer evoke tail movements (Fig. 13c). In control specimens with incisions of equal severity which left a Langerhans nerve intact, conduction peristed.

It is clear, therefore, that one function of the Langerhans bristle-bearing cells and the Langerhans axons, is to provide a route connecting the skin pulses with the caudal ganglion controlling the motor activity of the tail. It is not clear, however, whether the bristle-bearing cells themselves (assumed on morphological grounds to be sensory receptors) can initiate impulses in the Langerhans axons which will affect motor activity. Seeliger (1893) suggested that the bristles were embedded in the material of the house, and provided sensory information to the tail, perhaps sensing vibration of the house and triggering the animal's flight reaction (escape from house). We have made numerous attempts to alter the rhythmic activity of the tail by bending the bristles of these cells with micro needles. The bristles are relatively stiff, so that it is possible either to touch them near to the tip (when the basal region remains more or less static while the tip springs back from its bent position), or to bend the entire bristle by touching it near the base.

On one occasion only did we succeed in altering the rhythm of regular tail activity without inducing a skin pulse. On many other occasions, although skin pulses and tail movements could be evoked by touching the epithelium near the base of the bristle-bearing cell, touching the bristle itself gave no response.

It is difficult to assess such experiments, for although repeated trials (both with *O. labradoriensis* and *O. dioica*) gave negative results, it is possible that the incorrect stimulus was applied, or that the bristle had been damaged in some way—in the house the system may work as Seeliger (1893) suggested. However, since animals are often found in which the bristles are damaged (Fig. 6c), and since they are relatively much larger in juveniles (Galt, 1972), it is perhaps possible that in the adult animal it is no longer functional as a vibration or mechanoreceptor.

The coupling between skin pulses and the motor response, *via* the Langerhans receptor and caudal ganglion, is very close in fresh specimens so that each skin pulse evokes a motor response. In preparations which have been stimulated to yield many successive pulses, or in preparations treated with Mg^{++}, the skin pulses and the motor response may be uncoupled before Mg^{++} abolishes the motor response. Preliminary ultrastructural investigations of the Langerhans receptor suggest that the Langerhans nerves are axons of cells lying in the caudal ganglion, which synapse with the bristle bearing receptor cells themselves; persumably it is at this synapse that the system is uncoupled.

DISCUSSION

The observations reported above confirm the existence of propagated skin impulses in *Oikopleura,* providing another example of the same type of phenomenon known in siphonophores and hydromedusae (Mackie, 1965; 1970; Mackie and Passano, 1968), and in amphibian larvae (Wintrebert, 1904; Roberts, 1969; 1971; Roberts and Stirling, 1971).

In the hydromedusan *Sarsia,* a conducting epithelium such as the subumbrellar endoderm lamella conducts all-or-none impulses at velocities of 20–25 cm/sec (at 18° C). The wave forms recorded extracellularly are typically biphasic, with an initial positive component around 1 mv, and a refractory period of about 12 msec. The corresponding values for *Oikopleura* skin are 15–21 cm/sec (at 12–13° C), 1–2 mv, and 8–12 msec. In *Sarsia,* the conducting layer functions as part of the pathway whereby impulses from the excitable exumbrella epithelium reach the subumbrellar muscles whose contraction brings about a protective involution of the bell known as "crumpling." In *Oikopleura,* as in the amphibian larvae, the excitable skin acts as a part of the afferent sensory pathway by which stimulation of the external surface evokes impulses which travel to the central nervous system and trigger locomotory flight behavior.

It is a characteristic of epithelial conducting systems that they function in situations where the animal has to respond quickly by flight or by a protective response, to stimuli which may impinge upon it anywhere over a wide surface area. They provide a simple and effective (if crude) sensory adjunct to the nervous system. Conduction is diffuse and unpolarized, and no especial cells seem to be differentiated within the layer for conduction. The cells which conduct are the ordinary covering epithelial cells, and the enrollment of such cells into the response mechanism is manifestly efficient in terms of neuron economy.

In amphibians, the skin impulse system provides the tadpole with a precocious ability to respond to external stimuli (Wintrebert, 1904), being functional at a stage before the skin receives its definitive sensory innervation. During later development, this transitional device is lost, at different stages in different species (Roberts and Smyth, 1974). The skin impulses of amphibian larvae resemble those of *Sarsia* and *Oikopleura* in many respects, but conduction is somewhat slower (7.7 cm/sec in *Xenopus,* and 4.5 cm/sec in *Bufo*) and the impulses are of much longer duration, over 100 msec in *Xenopus* and up to 900 msec in *Bufo.* Roberts and Stirling (1971) suggest that this long duration is adaptive as it will set a long refractory period for the behavioral response, obviating useless flutter; no such consideration applies in the case of *Oikopleura* where the skin impulses are relatively very rapid events.

In *Xenopus,* the skin impulses are TTX-sensitive and appear to be generated across the inner membranes of the epithelial cells. The cells are electrically coupled, and impulses probably spread by direct current flow. Close appositions (gap junctions) are seen between adjacent cell membranes, presumably representing low resistance routes. Without intracellular recordings, it will not be possible to establish unequivocally that electrical spread occurs between cells in *Oikopleura,* but the existence of gap junctions between cells, and the all-or-none character of the spread is compatible with this interpretation, which is strongly suggested by the considerations listed earlier in the results section.

Larvaceans are interesting because they more closely resemble the larval stages of other tunicates than they do other adult tunicates; the group is generally accepted to have arisen by paedogenesis. Thus the larval tail, notochord, and other features of the ascidian or doliolid larvae are retained (Garstang, 1928). We have recently found that skin conduction occurs in the ascidian tadpole (Mackie and Bone, in preparation), and at least one group of chordates (anuran Amphibia) show skin conduction in the larval stage; perhaps we are concerned with a larval adaptation of possible widespread occurrence which has persisted in the adult *Oikopleura* as part of its paedogenic syndrome. Embryonic cells are frequently electrically coupled (*e.g.,* Bennett and Trinkaus, 1970), so that in a sense, larvae may be preadapted to non-nervous impulse conduction, utilizing it to enconomize in neuron number either until development of the definitive sensory apparatus takes place (amphibia), or to compensate for the drastic reduction in numbers of all cell types apparently consequent upon the production of large numbers of larvae (tunicata).

Considering the relatively small numbers of neurons and muscle cells involved, *Oikopleura* has quite a wide repertoire of patterns of tail movement. In intact animals, these are evidently under control of the caudal ganglion, linked with outside stimuli via the skin pulse system and the Langerhans axons. Salensky (1903) had inferred that the caudal ganglion must contain sensory as well as motor cell bodies, since he found somata of different sizes in the ganglion; it seems probable that the cells of origin of the Langerhans axons lie in the caudal ganglion, but most of the cells in the ganglion must be motor or associative. Our preliminary ultrastructural observations of the ganglion and caudal nerve cord have shown that there are both chemical synapses and what are presumably electrical junctions within the system, but consideration of the interconnection of cells will be deferred to a later account. The patterns of activity of the tail either involve short bursts of repetitive oscillation at regular intervals, or much longer periods of tail beating upon which a larger rhythm is superposed. This larger rhythm is apparently similar to that produced by surgery or by the action of drugs, when it appears in isolation. We may conclude that at least two types of repetitively firing cells are present in the caudal ganglion, and that they show different sensitivity to blocking agents.

In the lower chordates, such as the dogfish (Gray and Sand, 1936), where a regular swimming pattern is observed in spinal preparations after destruction of the brain, proprioceptive input is required to maintain the motor activity, which is, however, at constant frequency. Our observations have not enabled us to determine whether proprioceptive input is required for rhythmic activity by the caudal ganglion; certainly, the absence of movement in portions of tails lacking the caudal ganglion suggests that local reflexes involving segmental neuron patterns (of the type known in lower chordates and in amphioxus) are absent. Within the caudal nerve itself, there are cell bodies of two sizes, the larger giving rise to axons terminating in corymbiform motor endplates, the smaller to the branching beaded terminations (Fig. 2); there do not appear to be any internuncial or associative neuron cell bodies, as there are in the segmental neuron patterns of the lower chordate spinal cord. Histological evidence for the existence of sensory terminations associated with the caudal muscle cells is unconvincing; our ultrastructural observations suggest that both types of endings mentioned above are motor.

In *O. dioica* swimming bursts of symmetrical oscillation of isolated tails occasionally terminate with several tail beats to one side only; such an asymmetry does not suggest that a simple proprioceptive input maintains the usual oscillatory swimming pattern.

In some ways, the tail of *Oikopleura* offers one of the simplest systems utlizing the basic chordate characters of dorsal nervous system, segmental muscle blocks, and notochord to give oscillatory swimming movements but this simple system is different in several ways to that adopted by amphioxus and fishes. In the lower chordates, the nervous system is segmental in arrangement, linked patterns of segmental neurons control the oscillatory activity of the myotomes, and are dependent upon proprioceptive input. There are in all lower chordates, from amphioxus upwards, two basic types of muscle fiber in the myotomes, each utilized during different swimming patterns (Bone, 1966), and finally, so far as is known, bursts of oscillatory swimming controlled by higher centers are not observed. It is remarkable that with a small neuron number (some 40 cells in the caudal ganglion, and perhaps 50–60 in the caudal nerve), with only one type of muscle cell, and apparently without proprioceptive information, *Oikopleura* is able to oscillate its tail at different frequencies either continuously or in bursts.

We are grateful to Dr. C. L. Singla for assistance with electron microscopy and wish to thank the Director and staff of the Friday Harbor Laboratory for their hospitality. One of us (Q.B.) wishes to thank the University of Victoria, the Nuffield Foundation and the National Research Council of Canada for the award of a Nuffield-NRC visiting lectureship during the tenure of which this work was carried out. Financial support was provided by NRC grant A 1427 to G.O.M.

SUMMARY

The skin covering the tail and hinder trunk region of *Oikopleura* propagates impulses at 15–21 cm/sec. Spread is non-decremental and unpolarized. The impulses are of short duration (8–12 msec) and in general resemble skin impulses in hydromedusae more than those of amphibian and tunicate tadpole larvae.

The skin was examined by optical and electron microscopy. The cells are connected by gap junctions. Impulses are assumed to spread by direct current flow from cell to cell.

Electromyograms of tail activity during three behavior patterns (pumping, swimming, and house rudiment expansion) are analyzed in relation to neuromuscular histology. There appear to be at least two classes of pacemakers, both located in the caudal ganglion. There is no evidence that proprioceptive feedback is required for maintenance of rhythmic activity; and isolation of the tail from the trunk, which contains the cerebral ganglion, does not affect rhythmicity. The

system differs fundamentally from the locomotory systems of *Amphioxus* and fishes.

Skin pulses evoke swimming bursts in intact animals or briefly accelerate pre-existing rhythms. The conduction pathway from skin to caudal ganglion is shown to be a pair of nerves running from the latter to a receptor located in the skin at the back of the trunk. No other nerves end in the skin. Cutting these nerves blocks the response.

As in other cases, the conducting epithelium here functions as an extension of the afferent pathway, mediating an escape response following tactile stimulation.

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BIOTIC CENSUS OF CAPE COD BAY: HYDROIDS[1]

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The Biotic Census of Cape Cod Bay was initiated in 1965 by the Systematics-Ecology Program at the Marine Biological Laboratory, Woods Hole, Massachusetts. Completed in 1969, the census involved qualitative and quantitative sampling of the benthos to determine the species occurring in the bay, and to provide information about their ecology. This report is an account of the hydroids collected during the project.

The hydroids of the northeast have received greater attention than those from any other geographic area of North America. Over 75 papers bearing on the taxonomy and distribution of hydroids from New England and Atlantic Canada have been published since Stimpson's (1854) synopsis of the marine invertebrates of Grand Manan Island. Among those not already cited in Fraser's (1944) monograph are papers by Ruebush (1939), Crowell (1945, 1947), Fraser (1945), Berrill (1948), Merrill (1967), Bush and Zinn (1970), Templeman (1973), and Calder (1974), in which one or two species have been discussed. Others, including Préfontaine (1932), Prat (1933), Procter (1933), Dexter (1944, 1947), Fuller (1946), Woods Hole Oceanographic Institution (1952), Bousfield and Leim (1960), Brunel (1962, 1963, 1970), Préfontaine and Brunel (1962), Caddy (1970), and Bourget and Lacroix (1973), have included hydroids in a general biotic survey or in a study on fouling organisms. Vervoort (1972) included several species of hydroids from the northeast collected during cruises from the Lamont-Doherty Geological Observatory. A number of hydroid species from Newfoundland and Anticosti Island have been described by Leloup (1939, 1960), and papers on the hydroids of northern Canada by Calder (1970, 1972) included a number of species from the Strait of Belle Isle. Further sources of information on hydroids of the northeast include Fraser's (1946) book on distribution and relationship in American hydroids, and the identification manuals of Miner (1950), Smith (1964), and Gosner (1971). Despite this volume of work, little specific information is available concerning the hydroids of Cape Cod Bay.

The collection of hydroids from the biotic census was relatively small; Cape Cod Bay apparently does not have an especially rich hydroid fauna. Nevertheless, a number of interesting hydroids were present in the collection, particularly those species adapted to substrates of mud and sand.

The systematic arrangement used in this report differs significantly from that employed by Fraser (1944). While Fraser's contributions to North American hydroid taxonomy and zoogeography remain of monumental value, the system of classification in his 1944 monograph is little advanced over that used 75 years earlier. Unfortunately, no comprehensive treatise on the hydroids of North

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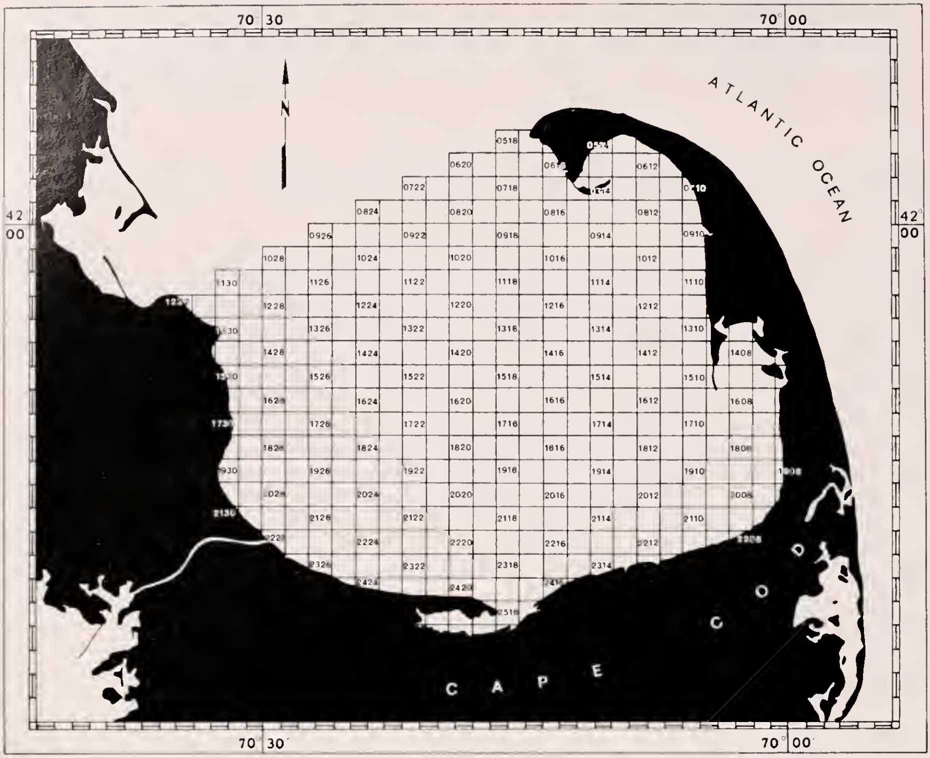
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FIGURE 1. Cape Cod Bay, showing the sampling quadrats of the biotic census.

America under a modern classification is available. For an appreciation of contemporary trends in hydrozoan classification, one must refer to papers such as those of Brinckmann-Voss (1968), Millard (1962, 1964, 1966), Rees (1956, 1957, 1966), Vervoort (1968, 1972), Hirohito (1974), and Leloup (1974).

MATERIALS AND METHODS

In establishing a sampling pattern for the Biotic Census, Cape Cod Bay was divided into a grid of one-mile squares; those quadrats chosen for sampling were assigned four-digit numbers (Fig. 1). Qualitative samples were taken within each designated quadrat using an epibenthic sled, a modified clam dredge, and a naturalist's dredge. Hauls were made toward the center from the corners of the quadrat. Quantitative samples were taken at each corner and from the center of each sampling quadrat using a 0.10 m^2 Smith-McIntyre grab. Qualitative and quantitative samples were sieved through a 1.0 mm screen. Specimens were relaxed in 0.015% propylene phenoxytol, fixed in 10% seawater formalin for 48 hours, and preserved in 85% ethanol.

Salinity and temperature data in Table I are based on bottom readings. Water temperature was recorded using a bathythermograph, and salinity samples were taken with a Van Dorn bottle. Depth data are given in Table I only for those areas of each quadrat where hydroids were collected.

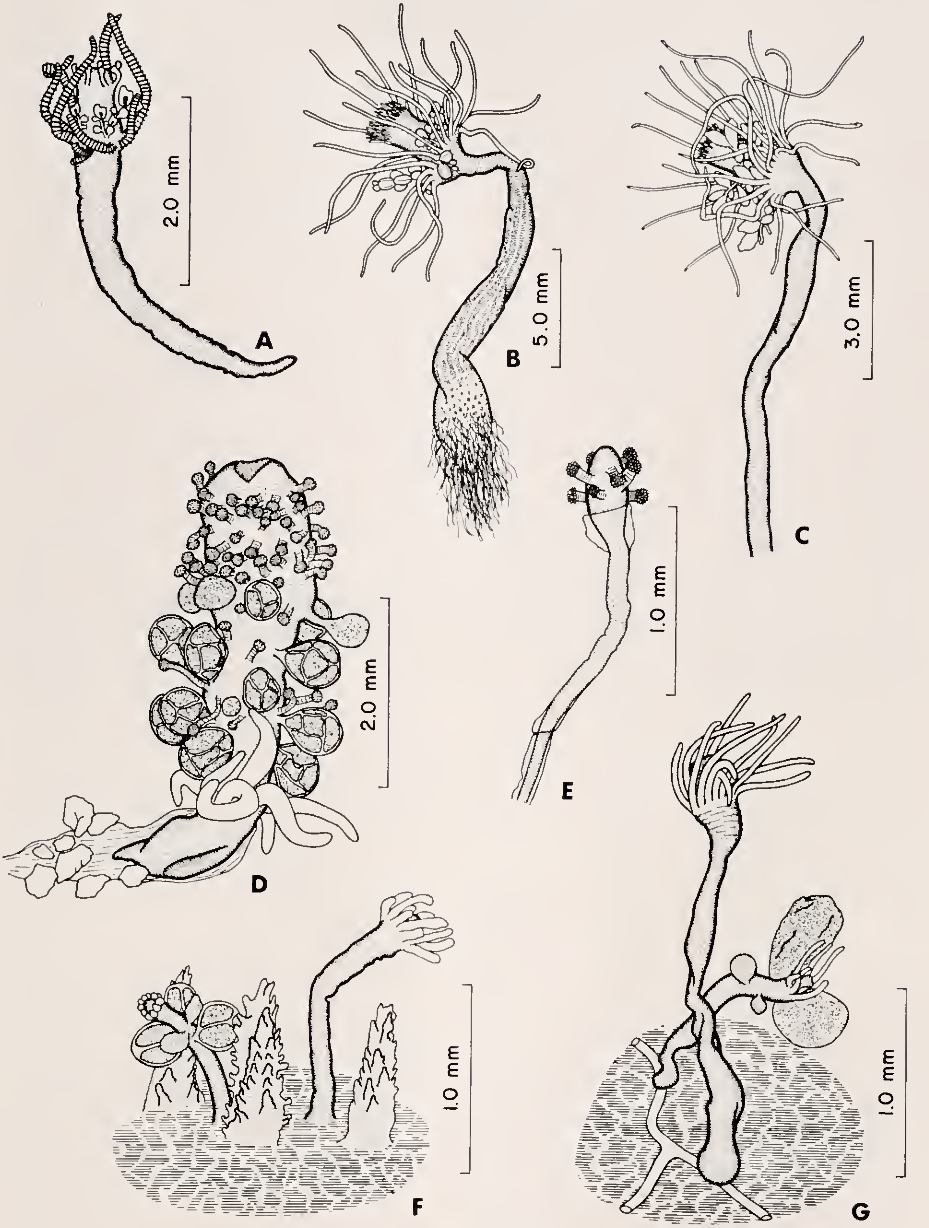
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FIGURE 2. Athecate hydroids from Cape Cod Bay. A shows *Euphysa farcta,* 1028; B, *Corymorpha pendula,* 1522; C, *Tubularia larynx,* 2314; D, *Acaulis primarius,* 1012; E, *Sarsia tubulosa,* 2028; F, *Hydractinia echinata,* 2318; G, *Stylactis arge,* 1808.

TABLE I

List of stations in Cape Cod Bay where hydroids were collected.

Station	Date	Depth (m)	Temperature (C)	Salinity ($^0/_{00}$)	Bottom type	Number species
0518	14-V-68	51–55	3.5	32.02	sand, silty sand	2
0616	18-VIII-69	8	21.6	31.25	sand	2
0620	18-IV-67	55	2.9	32.32	sand silt clay	1
0710	16-X-69	12	15.5	31.61	sand	2
0714	16-X-69	41	11.1	32.03	—	1
0718	6-I-69	50–54	3.1	32.55	silty sand	3
0824	28-VI-67	46–48	5.6	31.75	clayey silt, sandy silt	1
0910	19-VIII-69	9	22.7	31.20	gravel	1
0922	27-III-68	44	1.7	32.14	clayey silt	1
1012	7-IX-67	18–25	7.2	31.52	sand	6
1016	19-VIII-68	32–37	7.0	31.86	—	1
1028	21-IV-69	34–40	4.2	32.40	—	6
1114	11-III-69	31	1.5	32.48	—	1
1118	30-IX-68	40	4.4	31.97	—	1
1126	12-V-69	42	4.8	32.08	—	1
1130	22-I-68	15–35	0.7	31.84	sand, silty sand	7
1216	19-XII-67	32	6.5	31.67	clayey silt	1
1220	26-VIII-68	34	6.2	31.94	clayey silt	4
1228	10-V-67	27–33	4.8	31.47	silty sand	4
1232	16-X-69	12	14.0	31.74	sand	1
1310	10-IX-68	0	17.5	31.61	sand	1
1314	23-IV-68	30	5.0	31.99	clayey silt	2
1318	14-VI-67	33–35	5.7	31.58	clayey silt	3
1322	24-VII-67	34–38	4.8	31.78	clayey silt	3
1330	21-VII-69	6	12.9	31.35	rocky	2
1412	11-VI-68	11–14	7.0	31.53	sand	2
1416	1-X-68	29–30	9.5	31.90	sand silt clay	2
1428	13-V-69	25	4.8	32.04	silty sand	3
1510	11-IX-69	2–5	19.4	30.98	sand	3
1514	19-I-67	18	2.2	31.99	sand	2
1522	19-VIII-68	31–32	7.0	31.92	—	2
1526	21-I-69	24–34	1.6	32.47	silty sand	2
1530	21-XI-67	10	—	—	sand	2
1608	6-IX-67	2–4	18.7	30.44	sand	2
1612	13-V-69	2–9	12.0	30.16	sand	3
1616	22-IV-68	27	5.1	31.99	—	1
1624	13-V-68	32–33	5.5	31.49	sandy silt	4
1628	26-III-68	20–25	1.8	32.15	silty sand	8
1710	11-IX-69	3–6	19.5	31.18	—	2
1714	21-I-69	11	0.8	32.22	—	2
1718	29-X-68	26–28	13.1	31.99	silty sand	1
1808	19-VIII-69	3–5	23.5	31.21	sand	3
1812	20-II-68	2	1.0	31.96	sand	2
1816	13-V-69	15–19	6.5	31.45	sand	1
1820	1-X-68	25–27	7.1	31.96	—	2
1824	1-X-68	22	8.0	31.95	—	1
1828	11-III-69	13–23	1.5	32.28	silty sand	3
1910	17-V-67	5	10.5	30.86	sand	1
1914	18–XI-68	13	8.0	31.96	—	3
1918	4-VIII-66	21	6.8	31.92	sand	1
1922	14-VIII-67	23–26	6.6	31.73	silty sand	4

TABLE I (*continued*)

Station	Date	Depth (m)	Temperature (C)	Salinity (‰)	Bottom type	Number species
1926	19-XI-68	21	9.5	31.95	sandy silt	2
2008	23-IV-68	4–6	7.1	31.90	sand	2
2016	12-VI-68	16–18	8.1	31.33	sand	2
2024	27-III-68	23	1.8	32.20	—	1
2028	21-III-66	7	3.0	32.49	—	2
2110	11-IX-69	8–9	20.0	30.87	—	1
2114	25-X-67	7–13	11.5	31.53	sand	5
2118	15-X-68	17	13.6	31.90	sand	5
2126	7-II-66	21	1.0	—	—	1
2212	23-IV-68	3	5.2	31.89	sand	4
2220	7-I-69	16–17	1.5	32.12	—	3
2224	27-III-69	19	3.5	32.29	—	1
2314	18-VIII-69	8–10	20.4	31.36	—	2
2318	13-X-66	13–15	15.0	31.83	—	4
2326	21-VII-69	10–14	17.0	31.17	sand	2
2416	18-VIII-69	10	21.6	31.22	—	1
2420	18-VIII-69	8–10	17.0	31.45	sand	4

Descriptions, measurements, and illustrations in this report are based entirely on material from Cape Cod Bay. Terminology is largely as defined by Hyman (1940). The reported distribution refers to the North American Atlantic only. The synonymy list for each species includes the original author and any references relevant to the northeast not already cited by Fraser (1944). Representative specimens have been deposited in the Gray Museum, Marine Biological Laboratory, Woods Hole, Massachusetts.

SYSTEMATIC ACCOUNT

Order ANTHOMEDUSAE/ATHECATA
Suborder Capitata
Family Corymorphidae

Euphysa farcta (Miles, 1937)
Figure 2A

Dahlgrenella farcta Miles, 1937, p. 327, plates 1–4; Fraser, 1944, p. 102, fig. 75, 1945, p. 61, figs. 1–6.

Collection records: 0620, 0922, 1028, 1126, 1314, 1318, 1624, 1922.

Description: Polyps solitary, 1.5–9.0 mm high; hydrocaulus enclosed by a typically wrinkled and loosely-fitting tube of perisarc; perisarc extending a short distance over hydranth base; end of tube beyond tip of hydrocaulus occasionally frayed out into anchoring filaments. Hydranth club-shaped when extended, 0.8–1.7 mm long, 0.3–1.1 mm wide in preserved specimens; large specimens with as many as 25 or more papillae near point where hydranth merges with hydrocaulus. Tentacles in two whorls; oral tentacles 5–10 in number, small, capitate in smaller specimens, moniliform in larger ones; aboral tentacles moniliform, 8–17 in number. Buds and asexual bodies absent.

Medusa buds borne in small clusters on a whorl of short blastostyles arising just distal to aboral tentacles. Buds at first globular, later bell-shaped; tentacle bulbs four, one of these bearing a single tentacle.

Remarks: This hydroid was originally described as *Dahlgrenella farcta* by Miles (1937) from specimens collected in the Mount Desert Island region of Maine. Noting the similarity between hydroids of *D. farcta* and *Corymorpha aurata,* Rees (1938) placed *Dahlgrenella* in synonymy with *Corymorpha.* Later, *D. farcta* and *C. aurata* were removed to the genus *Euphysa* by Rees (1957). According to Kramp (1961), the only medusa of *Euphysa* ranging as far south as Cape Cod in the western Atlantic is *E. aurata.* Life history studies will probably reveal that *E. farcta* is a synonym of this species. Yet, hydroids in the present collection differed from descriptions of *E. aurata* by Rees (1938, 1946, 1957), Naumov (1960), and Brinckmann-Voss (1968) in having greater numbers of basal papillae. Cape Cod Bay specimens of *E. farcta* were typically much larger than the polyps of *Euphysa* sp. reported by Calder (1972) from Hudson Strait.

Reported distribution: Maine to Cape Cod Bay.

Corymorpha pendula L. Agassiz, 1862
Figure 2B

Corymorpha pendula L. Agassiz, 1862, p. 276, plate 26, figs. 7–17; Procter, 1933, p. 117; Dexter, 1944, p. 357; Fraser, 1944, p. 89, fig. 64; Dexter, 1947, p. 291; Bousfield and Leim, 1960, p. 13; Smith, 1964, p. 17; Brunel, 1970, p. 18.

Collection records: 0710, 0824, 1012, 1130, 1216, 1228, 1318, 1322, 1412, 1416, 1522, 1526, 1624, 1628, 1816, 1922, 2024, 2114, 2318.

Description: Hydroids solitary, ranging in height from 4–70 mm; hydrocaulus parenchymatous, coursed by branching and anastomosing gastrodermal canals; base of hydrocaulus bulbous with numerous papillae situated distal to a tangle of anchoring filaments; hydrocaulus tapering distally, narrowest just below hydranth; perisarc thin, closely fitting. Hydranth distinctly demarcated from hydrocaulus, reaching about 8 mm in length; tentacles all filiform, a single whorl of large aboral tentacles and a distal tuft of smaller, scattered oral tentacles; tentacle number varying with polyp size, about 50 aboral and 100 or more oral tentacles in larger specimens.

Blastostyles reaching about 10 mm long, arising just distal to aboral whorl of tentacles; gonophores sessile, oval, eumedusoid, with four radial canals frequently evident; tentacles four, rudimentary, occasionally difficult to discern.

Remarks: Descriptions by Agassiz (1862), Fraser (1944), and others indicate that the gonophores of *C. pendula* have one moderately large and three rudimentary marginal tentacles. Such tentacles were all vestigial in preserved material from Cape Cod Bay, and tentacles were frequently not discernable at all on the larger, ripe gonophores.

This species differs from *C. groenlandica,* reported from northern Canada (Calder, 1972), in having eumedusoid rather than cryptomedusoid gonophores. *C. glacialis,* occurring in the northeastern Atlantic, is distinguishable from *C. pendula* in lacking any trace of tentacles on the gonophores.

C. pendula was one of the most abundant hydroids in census collections, although most of the specimens were in rather poor condition.

Reported distribution: Gulf of St. Lawrence to Rhode Island.

Family Tubulariidae

Tubularia larynx Ellis and Solander, 1786

Figure 2C

Tubularia larynx Ellis and Solander, 1786, p. 31; Fraser, 1944, p. 99, fig. 72; Smith, 1964, p. 19; Brunel, 1970, p. 18.

Thamnocnidia spectabilis L. Agassiz, 1862, p. 271, plate 22, figs. 1–20.

Thamnocnidia tenella L. Agassiz, 1862, p. 275, plate 22, figs. 21–30.

Tubularia spectabilis Fraser, 1944, p. 100, fig. 73; Dexter, 1947, p. 291; Bousfield and Leim, 1960, p. 13.

Tubularia tenella Procter, 1933, p. 117; Fraser, 1944, p. 101, fig. 74; Brunel, 1963, p. 82; 1970, p. 18.

Collection records: 1012, 2314, 2318, 2326.

Description: Colonies growing in tangled masses reaching about 7 cm high. Hydrocaulus sinuous, irregularly branched, maximum diameter 0.7 mm; better-preserved specimens with bulb-like dilation at base of hydranth. Perisarc adherent, moderately thick, thinning out toward base of hydranth, usually twisted at origin of branches, extensively wrinkled, with annulations at irregular intervals. Hydranths small, reaching 3 mm high; tentacles filiform, 20–23 in an aboral whorl, these reaching 5 mm long; oral tentacles 15–20, shorter.

Gonophores sessile, oval, occasionally with 3–4 small, blunt, nematocyst-bearing tentacular rudiments evident; radial canals absent; actinulae produced. Blastostyles occasionally branched, arising just distal to aboral tentacles.

Remarks: Fraser (1944) included eight species of *Tubularia* from the Atlantic coast of Canada and the United States; several of these require comment. The account of *T. cristata,* quoted from McCrady's (1857) original description, indicates that the gonophores were deeply campanulate medusae, octagonal in cross-section and having eight meridional lines of "thread cells" (nematocysts) running up the exumbrella. From this description, and from the account of the hydroid, *T. cristata* McCrady, 1857 is regarded here as a synonym of *Ectopleura dumortieri* (van Beneden, 1844). An examination of Fraser's (1941) type specimens of *T. crassa* (U.S.N.M. No. 22746) revealed that the species is based on specimens of *Corymorpha pendula.* Two other species, *T. spectabilis* and *T. tenella,* were regarded as synonyms of *T. larynx* by Vervoort (1946). The status of *T. couthouyi* should also be subjected to close scrutiny.

T. larynx is distinguishable from *T. indivisa* in having branched stems with wrinkled and annulated perisarc, and gonophores with three to four tentacular rudiments and no radial canals. In *T. crocea,* the stems are generally unbranched and much less wrinkled and annulated than in *T. larynx.* Female gonophores of *T. crocea* have 6–10 laterally compressed ridges rather than tentacular rudiments.

Reported distribution: Newfoundland to Long Island Sound.

Family Acaulidae

Acaulis primarius Stimpson, 1854
Figure 2D

Acaulis primarius Stimpson, 1854, p. 10, plate 1, fig. 1; Procter, 1933, p. 117, fig. 29; Fraser, 1944, p. 87, fig. 62; Bousfield and Leim, 1960, p. 13; Smith, 1964, p. 17.

Collection records: 1012, 1016, 1118, 1220, 1322, 1416, 1522, 1612, 1624, 1820, 1922, 1926, 2016.

Description: Polyp solitary, vermiform, 1.0–11.0 mm high, occupying a small fraction of length of perisarcal tube; perisarc thin, loosely fitting, gelatinous, with adhering sand and silt particles, terminating below the filiform tentacles; end of tube occasionally frayed, forming anchoring filaments. Hydranth club-shaped, 0.7–9.0 mm long, having an irregular whorl of 8–13 large filiform tentacles proximally; remainder of hydranth with numerous scattered, capitate tentacles. Hydrocaulus reduced, conical, 0.3–3.0 mm long.

Gonophores sporosacs, borne in large numbers on short stalks among the capitate tentacles; ring canal, radial canals, and tentacles lacking.

Remarks: Thirty specimens of this unusual species were found in the samples from Cape Cod Bay. As with *Corymorpha* and *Euphysa,* hydroids of *Acaulis* are well-adapted for existence in a sandy or muddy substrate.

Reported distribution: Bay of Fundy to Cape Cod Bay.

Family Corynidae

Sarsia tubulosa (M. Sars, 1835)
Figure 2E

Oceania tubulosa M. Sars, 1835, p. 25, plate 5, fig. 11.

Syncoryna sarsii Loven, 1836, p. 275, plate 8, figs. 7–10.

Coryne mirabilis L. Agassiz, 1862, p. 185, plate 17, figs. 1–16; plate 18, figs. 1–25; plate 19, figs. 1–27.

Syncoryne mirabilis Leloup, 1939, p. 1, fig. 1; Fraser, 1944, p. 41, fig. 14.

Sarsia tubulosa Smith, 1964, p. 18.

Collection record: 2028.

Description: Hydroid fragmentary and small (2 mm high), with one hydranth. Perisarc of hydrocaulus occasionally wrinkled but not distinctly annulated, expanded into a thin, wrinkled covering over hydranth base. Hydranth oval, 560 μ long, 200 μ wide; hypostome dome-shaped; tentacles 10, scattered, capitate; terminal knobs about 70 μ wide.

Medusa buds absent.

Remarks: Identification of this small, sterile specimen was made after comparison with hydroids of *S. tubulosa* examined previously from Chesapeake Bay (Calder, 1971) and northern Canada (Calder, 1972). This is a cold water species; records of it as *Syncoryne mirabilis* in tropical and subtropical waters are almost certainly erroneous and are not included in the range below.

Reported distribution: Northern Canada to Chesapeake Bay.

Suborder Filifera
Family Hydractiniidae

Hydractinia echinata (Fleming, 1828)
Figure 2F

Alcyonium echinatum Fleming, 1828, p. 517.

Hydractinia polyclina Procter, 1933, p. 116.

Hydractinia echinata Fraser, 1944, p. 78, fig. 55; Crowell, 1945, p. 207; Dexter, 1947, p. 291; Bousfield and Leim, 1960, p. 13; Préfontaine and Brunel, 1962, p. 245; Smith, 1964, p. 18, plate 2, fig. 17; Merrill, 1967, p. 281.

Collection records: 1012, 1130, 1310, 1510, 1514, 1628, 1710, 1808, 1918, 1922, 1926, 2008, 2110, 2114, 2118, 2212, 2220, 2318, 2326, 2420.

Description: Colony with individual stolons distinguishable only in very young colonies. Typical colonies with encrusting base having numerous small prickles and large chitinous spines; spines reaching 1.4 mm high, consisting of a series of vertical, jagged ridges; coenosarc extending up between ridges. Gastrozooids columnar, arising individually from encrusting base, highly contractile, reaching 10 mm in length but usually less than 5 mm; manubrium conical. Tentacles filiform, number highly variable, generally between 10–25. When extended, longer, larger, upward-directed tentacles alternating with shorter, smaller, outward-directed ones. Slender dactylozooids occasionally present at aperture of shell, reaching 2.5 mm high; tentacles reduced to nematocyst-bearing knobs.

Gonozooids cylindrical, slender, reaching 4 mm in length but usually less than 1.5 mm; tentacles reduced to a series of knobs densely armed with nematocysts. Gonophores cryptomedusoid, occasionally as many as 10 or more developing concurrently on distal half of gonozooid.

Remarks: This well-known, eurythermal species was common in Cape Cod Bay on gastropod shells, most or all of which probably had been occupied by pagurid crabs. In addition to Fraser's (1944) distribution records, numerous collections of this species from Georges Bank and the coast off the middle Atlantic states were made by Merrill (1967).

Reported distribution: Labrador to Florida.

Stylactis arge Clarke, 1882
Figure 2G

Sytlactis (sic) *arge* Clarke, 1882, p. 138, figs. 18–20.

Stylactis sp. Crowell, 1947, p. 206.

Collection record: 1808.

Description: Hydroid with a slender, creeping hydrorhiza overlain by a thin sheet of perisarc; spines absent. Gastrozooids arising individually from hydrorhiza, reaching 3 mm high. Tentacles filiform, up to 20 in number, arising above a bulbous and somewhat rugose hydranth base; hypostome elongate.

Dactylozooids absent.

Gonozooids slender, up to 2.2 mm high; hydranth base neither bulbous nor rugose. Hypostome club-shaped; tentacles filiform, up to 10 in number. Medusa buds large, developing along distal quarter of gonozooid, too poorly preserved to permit description.

Remarks: In a previous report (Calder, 1971), this species was placed in the genus *Hydractinia* on the basis of studies by Kramp (1932), who suggested that

Stylactis merited no more than subgeneric rank. However, the differences in the structure of the hydrorhiza appear sufficient to warrant recognition of *Stylactis* as a valid genus. In fact, Kramp (1959, 1961) has retained the name *Stylactis*

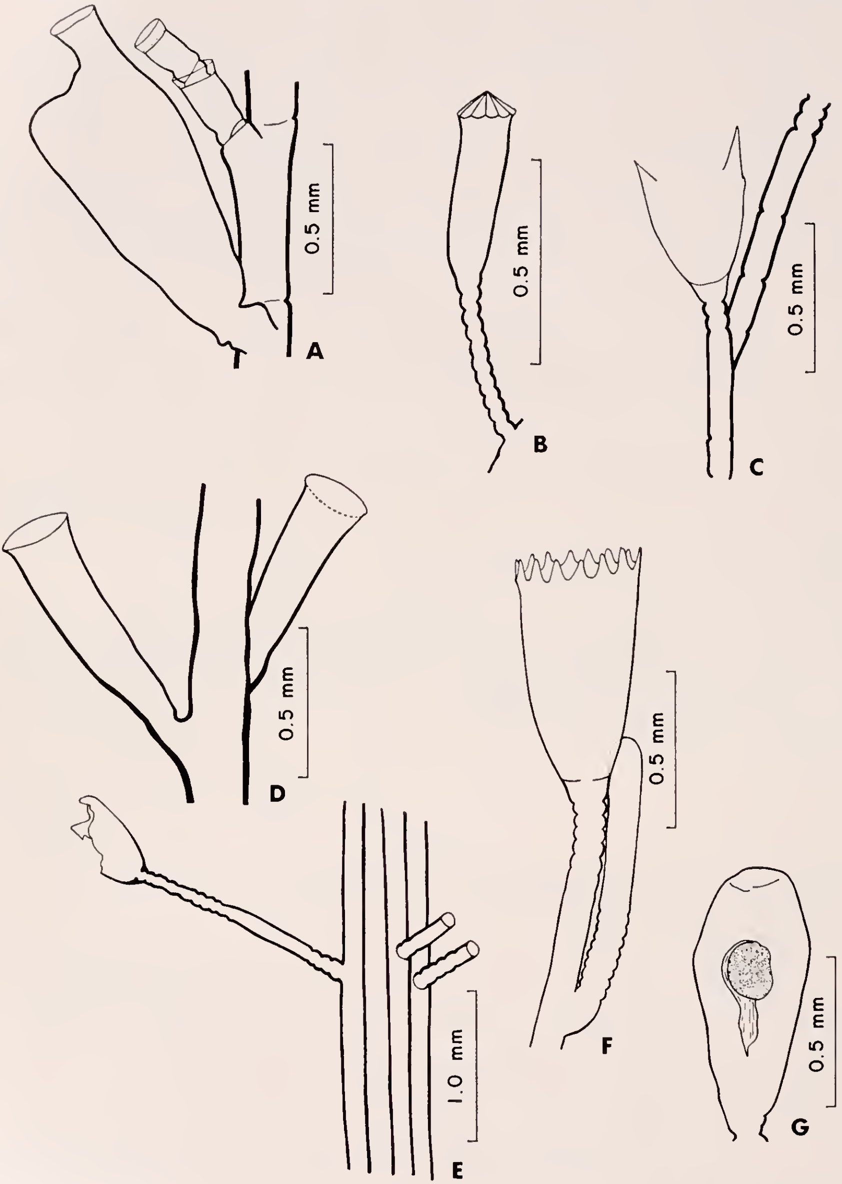

FIGURE 3. Thecate hydroids from Cape Cod Bay. A shows *Halecium halecinum*, 1322; B, *Calycella syringa*, 1130; C, *Lovenella gracilis*, 0616; D, *Lafoea dumosa*, 1028; E, *Campanularia verticillata*, 1530; F, *Clytia gracilis*, 1608; G, *Clytia gracilis*, gonotheca, 1608.

for the medusa stage of these hydrozoans. Bouillon (1971) discussed the status of the genus and summarized the characteristics of the various species described to date.

There is little apparent difference between descriptions of this species and *S. hooperi*. Asexual reproduction by hydranth autotomy has been observed in *S. arge* but not *S. hooperi;* small spines have been observed in *S. hooperi* but not *S. arge*. Medusae of the two species are inseparable from present descriptions. In both species the medusae are degenerate, short-lived, and released in greatest numbers at dusk (Clarke, 1882; Sigerfoos, 1899; Calder, 1971).

S. arge was represented in the present collection by a single colony.

Reported distribution: Cape Cod Bay to South Carolina.

Order LEPTOMEDUSAE/THECATA

Family Haleciidae

Halecium halecinum (Linnaeus, 1758)

Figure 3A

Sertularia halecina Linnaeus, 1758, p. 809.

Halecium gracile Verrill, 1874, p. 328.

Halecium halecinum Procter, 1933, p. 118; Fraser, 1944, p. 194, fig. 172; Bousfield and Leim, 1960, p. 14; Smith, 1964, p. 18, plate 2, figs. 18, 19; Vervoort, 1972, p. 25, fig. 3a.

Collection records: 1322, 1628, 1914.

Description: Hydrocaulus reaching 4.8 cm high, polysiphonic proximally, monosiphonic distally, divided into internodes at regular intervals by nodes sloping alternately from side to side. Each internode with a single, prominent, distal apophysis. Apophyses given off alternately from opposite sides of hydrocaulus at angles of less than 45°. Perisarc of hydrocaulus thick, annulations absent. Branches monosiphonic, arising from apophyses, arranged pinnately, divided into internodes at regular intervals by nodes that slope alternately from side to side. Length of internodes 632–828 μ, diameter at nodes 152–175 μ. Each internode with a single, distal apophysis. Apophyses arranged alternately on opposite sides of branch, each bearing a primary hydrotheca; secondary, tertiary and quaternary hydrothecae occasionally present. Branches occasionally rebranched; secondary branches arising from apophyses below primary hydrotheca. Primary hydrothecae frequently damaged or obliterated in present specimens; hydrophores of secondary, tertiary and quaternary hydrothecae relatively deep. All hydrothecae shallow, 35–58 μ deep; margin flaring but little, diameter at margin 135–163 μ, diameter at diaphragm 111–146 μ; distinct ring of puncta just distal to distinct diaphragm; pseudodiaphragm absent.

Single fertile colony female; gonothecae cone-shaped, 1109–1284 μ long to tip of neck; maximum width 478–548 μ; borne on branches. Pedicels short, not annulated, arising from apophyses adjacent to or in place of primary hydrothecae. Aperture at end of a short, somewhat flaring neck; neck situated at distal end and towards adcauline side of gonotheca.

Remarks: *Halecium halecinum* is similar to, but can be distinguished from, *H. beanii* in having a pinnate rather than a bushy colony form, and female gonothecae with a distal rather than a lateral aperture. Published descriptions indicate that the differences between *H. gracile* Verrill, 1874 and *H. halecinum* are minimal. Type specimens of *H. gracile* (U.S.N.M. 29076) differ from *H. halecinum* described here only in being somewhat less robust, and the two species are regarded as synonymous.

Fraser's (1944) record of this species (as *H. gracile*) from the Gulf of Mexico is considered doubtful.

Reported distribution: Labrador to Chesapeake Bay.

Family Calycellidae

Calycella syringa (Linnaeus, 1767)
Figure 3B

Sertularia syringa Linnaeus, 1767, p. 1311.

Calycella syringa Procter, 1933, p. 122; Fraser, 1944, p. 166, fig. 138; Bousfield and Leim, 1960, p. 14; Smith, 1964, p. 17, plate 2, fig. 14; Calder, 1970, p. 1516, plate 3, fig. 7.

Calicella syringa Vervoort, 1972, p. 36.

Collection record: 1130.

Description: Colony with a creeping hydrorhiza, giving rise to upright, unbranched pedicels of varying length; pedicels annulated in a close spiral throughout. Hydrothecae tubular, somewhat asymmetrical, 350–431 μ long from base to margin; margin slightly flaring, 123–146 μ in diameter; diaphragm absent. Operculum a folded cylindrical membrane forming a conical roof, separated from hydrothecal wall by a distinct, scalloped margin.

Gonophores lacking.

Remarks: *C. syringa* is very common in northeastern North America, yet it was found only once in the census collections.

Reported distribution: Northern Canada to Long Island Sound.

Family Lovenellidae

Lovenella gracilis Clarke, 1882
Figure 3C

Lovenella gracilis Clarke, 1882, p. 139, figs. 25–39; Fraser, 1944, p. 174, fig. 147.

Lovenella clausa Fraser, 1912, p. 5.

Collection records: 0616, 1510.

Description: Species represented by three young, unbranched colonies reaching a maximum height of 2.3 mm. Hydrocaulus monosiphonic, divided into rectangular internodes by more or less regularly located constrictions; diameter of hydrocaulus at nodes 76–93 μ. Perisarc of moderate thickness basally, thinner distally; that of the hydrotheca very thin. Annulations present only on hydranth pedicels and at base of hydrocaulus. Apophyses curving abruptly outward and upward beyond hydranth pedicels, giving hydroid a straight colony form. Hydrothecae turbinate, about 600 μ high, 300 μ wide, borne on short pedicels having two annulations; distance between hydrothecae about 1.2 mm. Operculum a folded continuation of hydrothecal wall, lacking any line of demarcation from rest of hydrotheca; number of opercular facets indeterminable. Diaphragm thin, basal chamber deep.

Gonothecae absent.

Remarks: Unlike the European *Lovenella clausa, L. gracilis* lacks a line separating the operculum from the rest of the hydrotheca; reports to the contrary are erroneous. In describing the species from Chesapeake Bay, Clarke (1882) neither reported observing a basal opercular line nor illustrated it in his figures. Specimens examined later from Chesapeake Bay also lacked any separation between hydrotheca and operculum (Calder, 1971). Specimens from Cape Cod Bay conformed to the descriptions given for material from the Chesapeake.

Reported distribution: Cape Cod Bay to North Carolina; northern Gulf of Mexico.

Family Lafoeidae

Lafoea dumosa (Fleming, 1828)

Figure 3D

Sertularia dumosa Fleming, 1828, p. 83.

Lafoea dumosa Procter, 1933, p. 123; Fraser, 1944, p. 221, fig. 205; Leloup, 1960, p. 221; Brunel, 1962, p. 40; 1970, p. 18.

Collection record: 1028.

Description: Hydrorhiza creeping, giving rise either to individual upright hydrothecae or hydrocauli. Hydrocauli short, up to 4 mm high, monosiphonic in present specimens; hydrothecae arising from all sides of hydrocaulus at an angle of less than 45°. Hydrothecae 700–839 μ long, somewhat asymmetrical, tapering gradually from margin to base, merging almost imperceptably with pedicel. Pedicel short, twisted or constricted beneath hydrotheca but not annulated; diaphragm lacking. Margin entire, often distinctly flaring, 210–256 μ wide. Perisarc of rhizocaulus, hydrocaulus, and hydrotheca thick.

Coppinia absent.

Remarks: Several studies have been made on morphological variability in species of *Lafoea*, the most recent by Vervoort (1972). Based on an examination of material from both the North and South Atlantic, Vervoort recognized *L. fruticosa* as distinct from *L. dumosa; L. gracillima* was considered a synonym of the former species.

Reported distribution: Northern Canada to the Caribbean Sea.

Family Campanulariidae

Campanularia verticillata (Linnaeus, 1758)

Figure 3E

Sertularia verticillata Linnaeus, 1758, p. 811.

Campanularia verticillata Fraser, 1944, p. 129, fig. 103; Bousfield and Leim, 1960, p. 14; Calder, 1970, p. 1519, plate 4, fig. 4.

Collection record: 1530.

Description: Specimen fragmentary, 1.1 cm long; rhizocaulus polysiphonic, 443–606 μ in diameter. Pedicels given off in verticils, most broken off near base, annulated throughout distal half and at base; a distinct ball-like annulation occurring just below hydrotheca. Hydrotheca with an annular thickening basally; basal chamber small.

Gonothecae absent.

Remarks: Despite the poor condition of this specimen, it was readily distinguishable as *C. verticillata* in possessing a polysiphonic rhizocaulus with the pedicels arranged in verticils.

Reported distribution: Northern Canada to Long Island Sound.

Clytia gracilis (M. Sars, 1851)
Figure 3F, G

Laomedea gracilis M. Sars, 1851, p. 138.

Gonothyraea gracilis Fraser, 1944, p. 148, fig. 121.

Collection records: 1608, 1808.

Description: Colony consisting of a creeping hydrorhiza and upright, branched or unbranched pedicels. Branched colonies up to 10 mm high; unbranched pedicels plus hydrotheca 5 mm high. Pedicels annulated proximally and distally, smooth elsewhere. Branches resembling primary pedicel and directed immediately upward from a short, curved apophysis situated a short distance below the hydrotheca of the pedicel from which it arises. Hydrothecae cone-shaped, 560–851 μ long, 327–385 μ wide at margin. Margin with about 14 sharp, deeply-cut teeth separated by U-shaped incisions. Diaphragm thin; basal chamber of moderate size, cup-shaped. Perisarc thin.

One club-shaped gonotheca containing a single medusa bud present, 950 μ long, 400 μ wide, truncate distally; walls smooth, perisarc thin, pedicel short.

Remarks: Hydroids of this species are known to produce a free medusa stage (Stechow, 1923a; Vervoort, 1972; Sandifer, Smith, and Calder, 1974). These medusae possess four tentacles at liberation, indicating that this hydrozoan belongs in the genus *Clytia*. Rees and Thursfield (1965) regarded this species as a synonym of *C. hemisphaerica*.

Reported distribution: Nova Scotia to the Caribbean Sea.

Clytia hemisphaerica (Linnaeus, 1767)
Figure 4A, B

Medusa hemisphaerica Linnaeus, 1767, p. 1098.

Campanularia johnstoni Alder, 1856, p. 359, fig. 8.

Clytia johnstoni Fraser, 1944, p. 138, fig. 111; Smith, 1964, p. 17, plate 2, fig. 4.

Collection records: 2314, 2416.

Description: Pedicels arising from a creeping hydrorhiza; colony either unbranched or branched, with as many as 6 branches present. Branched colonies reaching 12 mm high; unbranched pedicels plus hydrotheca up to 7 mm high,

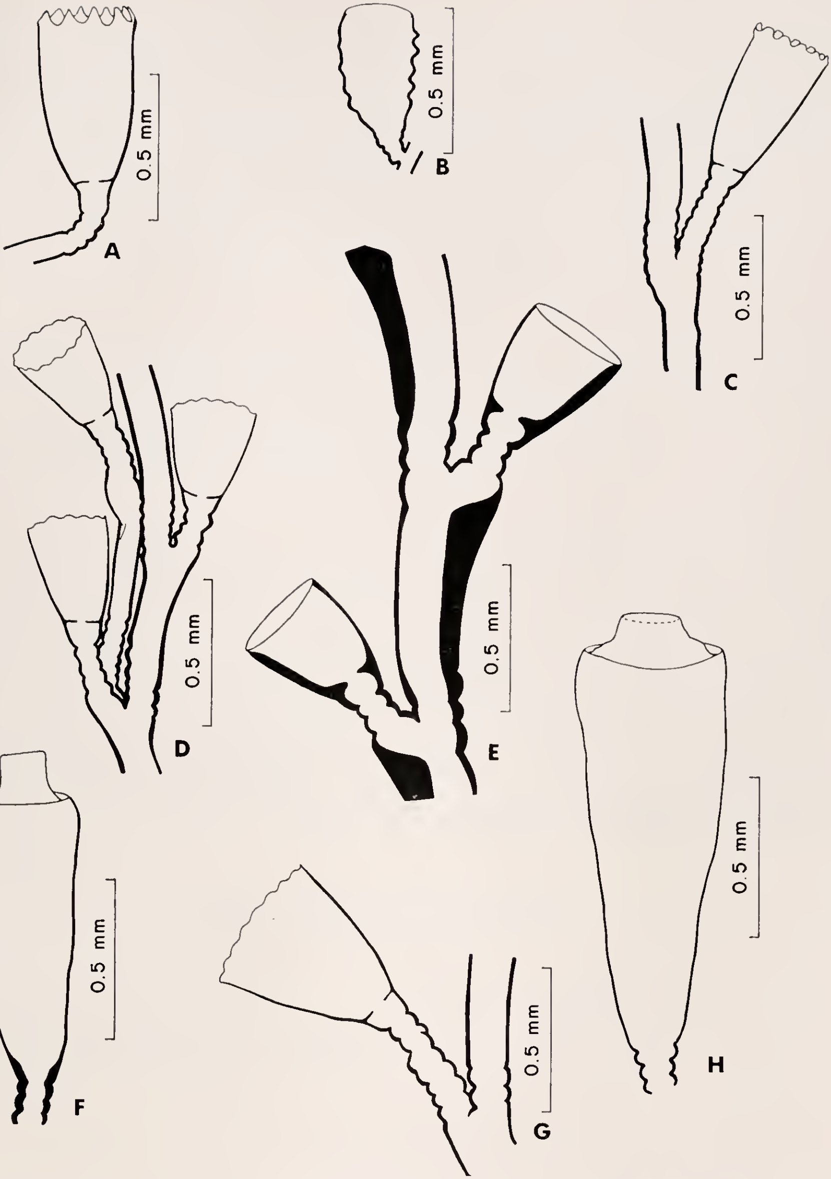

FIGURE 4. Thecate hydroids from Cape Cod Bay. A shows *Clytia hemisphaerica,* 2314; B, *Clytia hemisphaerica,* gonotheca, 2314; C, *Gonothyraea loveni,* 1910; D, *Obelia dichotoma,* 1012; E, *Obelia geniculata,* 1228: F, *Obelia geniculata* gonotheca, 2420; G, *Obelia longissima,* 1318; H, *Obelia longissima,* gonotheca, 0518.

One, two, or even three branches may arise from a single pedicel or branch; each branch resembling primary pedicel and directed immediately upward from a short, curved apophysis located a short distance below hydrotheca of pedicel from which it arises. Primary pedicels annulated at origin from hydrorhiza and below hydranth, typically smooth or with a few irregular annulations or wrinkles elsewhere; branches annulated above apophysis and below hydrotheca, usually smooth elsewhere. Cone-shaped hydrothecae 525–816 μ deep, 292–373 μ wide at margin, with walls slightly convex above diaphragm. Margin with about 10–14 sharply pointed, triangular, deeply-cut teeth separated by semicircular incisions. Diaphragm thin; basal chamber relatively large, cup-shaped. Perisarc of moderate thickness, being somewhat thicker proximally than distally on colonies.

Gonothecae about 600 μ high, 300 μ wide, arising from a short pedicel on upright parts of colony or hydrorhiza. Distal end truncate; walls strongly ribbed in a close spiral, resembling a chinese lantern.

Remarks: *Clytia johnstoni* has been known for some time to be the hydroid of the medusa *Phialidium hemisphaericum.* Rees and Thursfield (1965) and Millard (1966) united the two stages under the name *Clytia hemisphaerica.* Millard observed that the generic name *Clytia* Lamouroux, 1812 predates *Phialidium* Leuckart, 1856, while the specific name *hemisphaerica* Linnaeus, 1767 predates the name *johnstoni* Alder, 1856, and she designated *C. hemisphaerica* as the type species of the genus *Clytia.*

This is a morphologically variable species, as noted by Ralph (1957). She found that hydroids of *C. johnstoni* along the New Zealand coast varied widely in colony height and hydrotheca length and tooth shape, as well as in height and degree of annulation of the gonotheca. Various forms of Ralph's specimens resembled descriptions of *C. cylindrica, C. edwardsi,* and *C. minuta,* and she suggested that the three species may prove to be synonyms of *C. johnstoni.* To this list of probable synonyms Millard (1966) added *C. gracilis,* but Vervoort (1968) did not concur with this, having never seen intermediates between the two. He observed that the hydrotheca of *C. gracilis* is undulating in cross-section and that the gonotheca is invariably smooth, unlike *C. hemisphaerica.*

Reported distribution: Labrador to Florida.

Gonothyraea loveni (Allman, 1859)
Figure 4C

Laomedea loveni Allman, 1859, p. 138.

Gonothyraea loveni Préfontaine, 1932, p. 206; Procter, 1933, p. 121; Fraser, 1944, p. 149, fig. 123; Bousfield and Leim, 1960, p. 14; Préfontaine and Brunel, 1962, p. 245; Smith, 1964, p. 18; Calder, 1970, p. 1520, plate 4, fig. 6.

Collection records: 0910, 1012, 1028, 1220, 1330, 1514, 1812, 1910, 2008, 2114, 2118, 2420.

Description: Colonies reaching 1.7 cm high, growth sympodial; hydrocaulus monosiphonic; perisarc variable in thickness. Internodes of moderate length, each annulated basally and with an apophysis distally. Apophyses alternating from side to side, bearing hydrothecal pedicels, branches, or both; branches resembling hydrocaulus. Pedicel length variable but typically short and annulated throughout. Hydrothecae cone-shaped, 489–804 μ long, 245–443 μ wide at margin; marginal teeth truncate or bluntly rounded, occasionally with a slight notch medially and separated by relatively shallow incisions. Diaphragm thin, basal chamber usually large.

Gonothecae absent.

Remarks: Specimens of this species were readily identified, despite the absence of gonothecae, on the basis of their distinctive tooth shape. While the teeth were slightly notched in some specimens, none showed the median furrow that bisects each tooth in hydrothecae of *G. hyalina.*

Known distribution: Northern Canada to Chesapeake Bay.

Obelia dichotoma (Linnaeus, 1758)

Figure 4D

Sertularia dichotoma Linnaeus, 1758, p. 812.

Obelia dichotoma Préfontaine, 1932, p. 206; Procter, 1933, p. 120; Fraser, 1944, p. 155, fig. 127; Fuller, 1946, p. 153; Bousfield and Leim, 1960, p. 14; Préfontaine and Brunel, 1962, p. 245; Brunel, 1970, p. 18.

Laomedea dichotoma Leloup, 1960, p. 222.

Collection records: 1012, 1510, 1608, 1710.

Description: Sympodial colonies reaching 1 cm high above a creeping hydrorhiza. Hydrocaulus monosiphonic, zigzag; internodes relatively short, each with several annulations proximally and an apophysis distally. Apophyses alternating from side to side, each supporting a hydrothecal pedicel. Branches, when present, arising from apophyses near origin of hydrothecal pedicels; branch internodes resembling those of hydrocaulus. Hydrothecal pedicels with 2–10 annulations; developing branch internodes resembling pedicels but smooth in middle. Hydrothecae cone to bell-shaped, 327–431 μ deep, 265–315 μ wide at margin; margin with numerous low, rounded teeth; diaphragm thin, transverse or distinctly oblique; basal chamber usually small and wide relative to its height.

Gonothecae elongate, about 750 μ long and 300 μ wide, truncate distally with a terminal collar; perisarc not distinctly thickened basally. Pedicels short, annulated throughout.

Remarks: This species matures and regresses at a much smaller size than *O. longissima,* and it is improbable that the two are synonymous as Naumov (1960) indicated. The hydrothecae of *O. dichotoma* are also distinctly smaller than those of *O. longissima.*

Millard (1966) considered *O. dubia,* described by Nutting (1901) from Alaska, to be synonymous with *O. dichotoma.*

Reported distribution: Newfoundland to the Gulf of Mexico.

Obelia geniculata (Linnaeus, 1758)

Figure 4E, F

Sertularia geniculata Linnaeus, 1758, p. 812.

Obelia geniculata Préfontaine, 1932, p. 206; Procter, 1933, p. 120; Fraser, 1944, p. 158, fig. 130; Bousfield and Leim, 1960, p. 14; Préfontaine and Brunel, 1962, p. 246; Smith, 1964, p. 18.

Collection records: 1228, 1330, 1412, 1612, 1828, 2028, 2114, 2212, 2420.

Description: Colony sympodial, reaching 1.3 cm high, arising from a creeping hydrorhiza. Hydrocaulus monosiphonic, zigzag, unbranched or irregularly branched, divided into internodes 548–1050 μ long, 175–489 μ wide at apophyses. First internode with 2–4 annulations at juncture with hydrorhiza; remaining internodes with 0–4 annulations at proximal end. Perisarc very thick, especially on side bearing hydrotheca, forming a distinctive, knee-like projection into lumen of internode just below hydrotheca. Each internode with a distal apophysis; apophyses given off alternately from side to side, each supporting a hydrotheca *via* a pedicel consisting of from 2–10 annulations. Hydrotheca cup-shaped, depth 268–525 μ, width at margin 245–443 μ, margin entire; perisarc thick, particularly along abcauline wall, and a prominent annular thickening basally; basal chamber of moderate size.

Gonothecae lanceolate, arising from apophyses *via* short pedicels having 2–4 annulations; distal end truncate, provided with a narrow collar. Perisarc of moderate thickness except basally where a distinct thickening occurs. Gonotheca length 735–1167 μ, maximum width 256–466 μ.

Remarks: Variation in the colony form of *O. geniculata* was studied by Ralph (1956), who observed differences in size and degree of branching from different latitudes along the New Zealand coast. Variability in hydrotheca and internode morphology was discussed and illustrated by Leloup (1974). Specimens from Cape Cod Bay varied widely in internode length and width, hydrotheca size, perisarc thickness, and degree of annulation. In none of the specimens was the perisarc as thin as that observed in hydroids of *O. geniculata* from Chesapeake Bay (Calder, 1971).

Reported distribution: Northern Canada to the Caribbean Sea.

Obelia longissima (Pallas, 1766)
Figure 4G, H

Sertularia longissima Pallas, 1766, p. 119.

Obelia longissima Préfontaine, 1932, p. 206; Procter, 1933, p. 120; Fraser, 1944, p. 162, fig. 133; Préfontaine and Brunel, 1962, p. 246; Bourget and Lacroix, 1973, p. 872.

Obelia flabellata Fraser, 1944, p. 157, fig. 129.

Laomedea longissima Leloup, 1960, p. 222.

Laomedea (Obelia) longissima Vervoort, 1972, p. 93, fig. 27.

Collection records: 0518, 1220, 1318, 1624, 1628, 1820, 2212, 2420.

Description: Colony sympodial, reaching 15 cm high and regularly branched; hydrocaulus monosiphonic. Internodes very long, bearing several distinct annulations proximally and a single apophysis distally; apophyses alternating from side to side. Perisarc thick, becoming progressively thicker and darker in color towards older portions of colony. Apophyses giving rise to a hydrothecal pedicel initially, and later to one or more branches. Branches similarly divided into internodes and rebranched in like fashion. Hydrothecal pedicels of varying length, usually annulated throughout. Hydrothecae cone-shaped, 572–793 μ deep, 385–501 μ wide at margin, margin undulating; diaphragm thin, transverse or occasionally somewhat oblique. Basal chamber of varying size but usually moderately large and cup-shaped.

Gonothecae lanceolate, 992–1656 μ long, 420–560 μ wide, borne on short, annulated pedicels arising from apophyses or in one colony directly from hydrothecal pedicels; distal end truncate with a small terminal collar. Perisarc relatively thin, not provided with any distinct basal thickening.

Remarks: *Obelia flabellata* Hincks, 1866, reported from Cape Cod Bay by Fraser (1944), is a synonym of *O. longissima* (Bonnevie, 1899; Broch, 1918; Naumov, 1960).

Colonies of this species often attain considerable length, sometimes reaching 50–60 cm (Fraser, 1944).

Known distribution: Northern Canada to Chesapeake Bay.

Family Sertulariidae

Dynamena pumila (Linnaeus, 1758)

Figure 5A

Sertularia pumila Linnaeus, 1758, p. 807; Préfontaine, 1932, p. 206; Prat, 1933, p. 107; Procter, 1933, p. 123; Dexter, 1944, p. 356; Fraser, 1944, p. 286, fig. 274; Dexter, 1947, p. 291; Préfontaine and Brunel, 1962, p. 246; Smith, 1964, p. 18, plate 2, fig. 10.

Dynamena pumila Leloup, 1939, p. 6, fig. 4; Calder, 1970, p. 1528, plate 6, fig. 1.

Sertularella pumila Bourget and Lacroix, 1973, p. 872.

Collection records: 1114, 1130, 1314, 1428, 1628, 1718, 1824, 2118, 2126, 2220.

Description: Colonies largely fragmentary, largest reaching 7 mm high; hydrocaulus monosiphonic. Two very short athecate internodes basally, their nodes oblique with end of one internode extending onto next and tapering to a point. Remaining internodes thecate, bearing 1–2 pairs of hydrothecae; internode length with one pair of hydrothecae 466–758 μ, with two pairs 1085–1307 μ. Only one colony branched; branch arising just below a hydrotheca on proximal portion of internode, passing outward almost perpendicularly to axis of hydrocaulus. Hydrothecae opposite, well-separated front and back, nearly cylindrical and curving outward. Abcauline wall concave, 187–292 μ long; adcauline wall convex, 350–501 μ long. Base of hydrotheca 129–198 μ wide in profile. Hydrothecal aperture large; margin with two prominent lateral teeth and a smaller median adcauline tooth; operculum of two valves, abcauline valve larger.

Gonothecae absent.

Remarks: *D. pumila* typically occurs in the intertidal zone, and all the specimens in the present collection consisted of empty skeletons. This is a common species in boreal waters on both sides of the Atlantic.

Reported distribution: Labrador to New Jersey.

Hydrallmania falcata (Linnaeus, 1758)

Figure 5B

Sertularia falcata Linnaeus, 1758, p. 910.

Hydrallmania falcata Préfontaine, 1932, p. 206; Procter, 1933, p. 124; Fraser, 1944, p. 250, fig. 236; Bousfield and Leim, 1960, p. 14; Leloup, 1960, p. 223; Préfontaine and Brunel, 1962, p. 245.

Collection records: 1228, 1828.

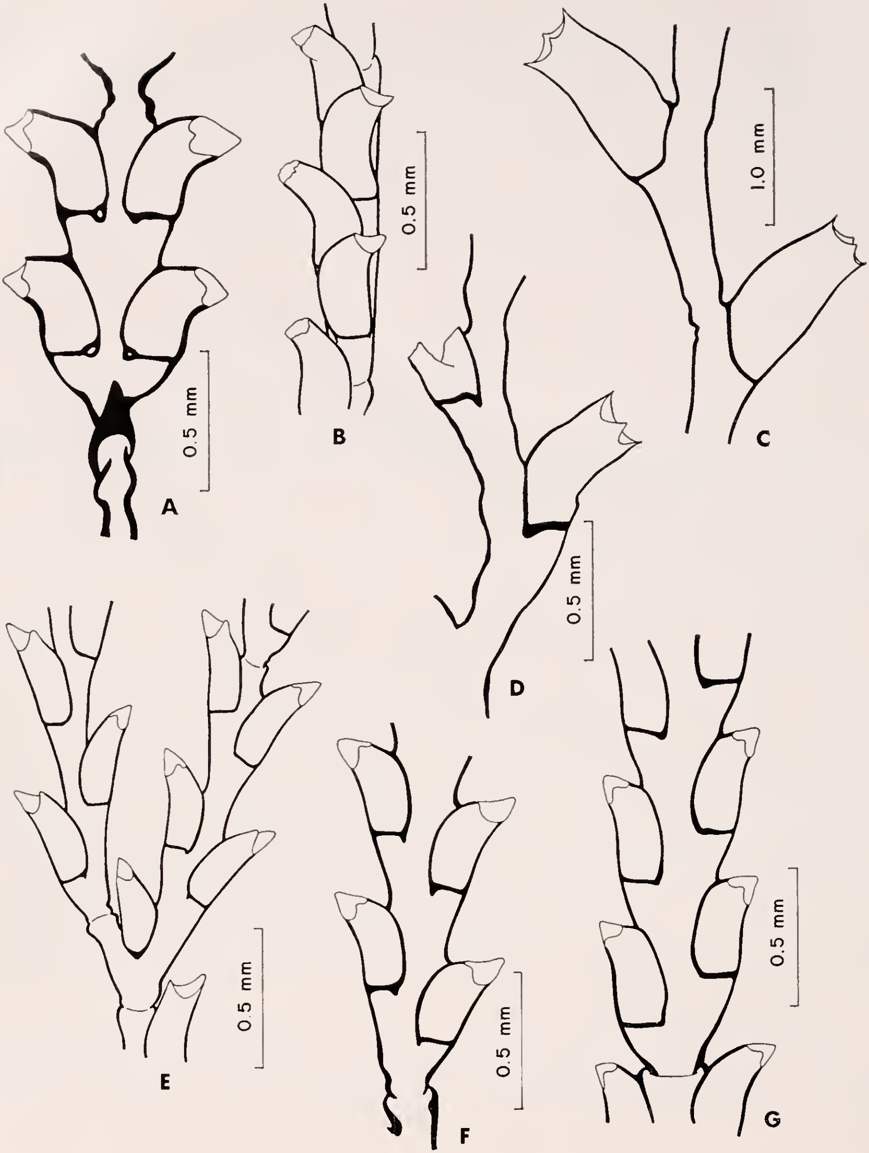

FIGURE 5. Thecate hydroids from Cape Cod Bay. A shows *Dynamena pumila,* 1628; B, *Hydrallmania falcata,* 1228; C, *Sertularella polyzonias,* 1028; D, *Symplectoscyphus tricuspidatus,* 0718; E, *Sertularia argentea,* 1616; F, *Sertularia cupressina,* 2224; G, *Sertularia latiuscula,* 2114.

Description: Species represented by two fragmentary branches having 4 and 24 hydrothecae respectively. Nodes distinct, oblique; internodes varying in length, each with 3–4 hydrothecae. Hydrothecae flask-shaped, crowded in a single row; margins oval, turned alternately from side to side; abcauline wall 420–466 μ long from base to tip of tooth; teeth two; operculum of one valve.

Gonothecae absent.

Remarks: Fully-developed colonies of this species are easily identified because the hydrothecae occur on one side of the branch only. However, in younger colonies, the hydroid has alternately-placed hydrothecae in two rows on the branches (Broch, 1918), and could easily be mistaken for a species of *Sertularia*. Broch observed that such colonies were sometimes distinguishable as *Hydrallmania* in having hydrothecae with the adcauline sinus deeper than the abcauline.

H. falcata is common in boreal waters of the North Atlantic, and has some commercial importance in parts of Europe, being sold as "coral moss" or "white weed" for decorative purposes (Hancock, Drinnan and Harris, 1956; Naumov, 1960).

Reported distribution: Southern Labrador to Long Island Sound.

Sertularella polyzonias (Linnaeus, 1758)

Figure 5C

Sertularia polyzonias Linnaeus, 1758, p. 813; Brunel, 1970, p. 18.

Sertularella polyzonias Préfontaine, 1932, p. 206; Procter, 1933, p. 124; Fraser, 1944, p. 268, fig. 258; Leloup, 1960, p. 223; Préfontaine and Brunel, 1962, p. 246; Caddy, 1970, table 1, p. 1.

Sertularella gigantea Fraser, 1944, p. 264, fig. 250.

Collection records: 1028, 2118.

Description: Unbranched or irregularly branched colonies reaching 2 cm high with monosiphonic, slightly zigzag hydrocaulus; branches similar to hydrocaulus, arising from internode just below hydrotheca. Hydrocaulus and branches marked at regular intervals by oblique nodes sloping alternately in opposite directions; diameter at nodes 223–326 μ. Internodes 1000–1567 μ long, each with a single, distally-located hydrotheca. Hydrothecae alternate, walls smooth or with a few faint transverse wrinkles. Abcauline wall 1062–1190 μ long; length adcauline wall free 921–1062 μ; total depth 1073–1167 μ. Hydrothecal margin opening quadrate, 454–489 μ wide, occasionally renovated with four distinct and equal teeth. Operculum of four faintly striated, triangular valves, closing to form a pyramid-shaped lid. Internal submarginal teeth absent. Perisarc thick, annulated at base of hydrocaulus and branches, bulging above node at proximal end of each internode.

Gonothecae absent.

Remarks: *S. polyzonias* occurs in a robust and a fine growth form (Broch, 1918; Vervoort, 1946). The former has been recognized by several authors (Nutting, 1904; Fraser, 1944; Naumov, 1960) as a distinct species, *S. gigantea* Mereschkowsky, 1878.

Reported distribution: Northern Canada to Georgia.

Symplectoscyphus tricuspidatus (Alder, 1856)

Figure 5D

Sertularia tricuspidata Alder, 1856, p. 356, plate 13, figs. 1, 2.

Sertularella tricuspidata Préfontaine, 1932, p. 206; Procter, 1933, p. 124; Fraser, 1944, p. 274, fig. 264; Bousfield and Leim, 1960, p. 14; Leloup, 1960, p. 224; Préfontaine and Brunel, 1962, p. 246; Calder, 1970, p. 1531; plate 6, figs. 7, 8.

Symplectoscyphus tricuspidatus Vervoort, 1972, p. 166, fig. 54.

Collection record: 0718.

Description: Specimen fragmentary, unbranched, 3.2 mm high, bearing six hydrothecae. Hydrocaulus monosiphonic, zigzag, 117–152 μ wide at nodes; internodes 408–525 μ long, each with a single hydrotheca. Hydrothecae alternate, all but one damaged. Intact hydrotheca long, tubular, smooth and curved outward. Abcauline wall concave, length to tip of tooth 385 μ; adcauline wall convex, adnate portion 245 μ long, free portion to tip of tooth 420 μ. Margin 221 μ wide, parallel to axis of internode; teeth three, separated by deep sinuses; internal submarginal teeth absent. Operculum and hydranth missing.

Gonothecae absent.

Remarks: The genus *Symplectoscyphus* Marktanner-Turneretscher, 1890 is recognized here for those species of Sertulariidae, formerly placed in the genus *Sertularella,* that have three marginal teeth. The splitting of *Sertularella* into two genera was proposed by Stechow (1923b), and has been adopted by Ralph (1961), Millard (1964, 1967, 1968, 1971), Vervoort (1972). Leloup (1974), and others.

S. tricuspidatus is a common hydroid in boreal and arctic waters but was collected only once during the Biotic Census of Cape Cod Bay.

Reported distribution: Northern Canada to New Jersey.

Sertularia argentea Linnaeus, 1758
Figure 5E

Sertularia argentea Linnaeus, 1758, p. 809.

Thuiaria argentea Procter, 1933, p. 124; Fraser, 1944, p. 293, fig. 280; Smith, 1964, p. 18.

Sertularia cupressina f. *argentea* Leloup, 1960, p. 224.

Collection records: 1130, 1616, 1628, 2212.

Description: Hydroids all either small or fragmentary, the largest reaching 4 cm high. Hydrocaulus monosiphonic, divided at more or less regular intervals by distinct nodes. Internodes of moderate length with one, two, or three branch-bearing apophyses, each apophysis with an axillary hydrotheca; occasional internodes with an additional pair of subopposite hydrothecae distally. Perisarc thick and horn-colored in older portions of colony. Branches relatively short, dichotomously rebranched, given off alternately from opposite sides of the hydrocaulus or occasionally tending towards a spiral arrangement. Branches in all but young colonies twisted basally, the broad surface being horizontal, divided into internodes having from one to three pairs of hydrothecae. Hydrothecae slender, fusiform, subopposite to subalternate. Distance between hydrothecae on same side of a given internode 187–478 μ. Hydrothecae immersed somewhat over half their length, with distal portion curving outward and upward. Abcauline wall concave, 233–327 μ long; adcauline wall convex, 187–280 μ adnate, 117–198 μ free. Margin with two prominent teeth; operculum of two unequal valves.

Gonothecae arrowhead-shaped, 1.1–1.5 mm long from base to rim of orifice, arising from upper surface of branches near bases of hydrothecae. Distal end with two large spines; orifice round, at end of short collar; well-developed submarginal teeth present.

Remarks: Maturo (1973) observed that intercolony variation in bryozoans causes difficulties for the systematist, who must decide whether such specimens represent separate species, a single broadly variable species, or a single variable species having several varieties or forms. This dilemma is also frequently encountered by the hydroid systematist; the genus *Sertularia* with its species *S. argentea* and *S. cupressina* represents an example. The two have been regarded as distinct species by some authors (Hincks, 1868; Nutting, 1904; von Reitzenstein, 1913; Fraser, 1944; Hancock, Drinnan and Harris, 1956; Rees and Thursfield, 1965; Calder, 1971) and synonyms by others (Broch, 1918; Kramp, 1929, 1938; Vervoort, 1946, 1972; Naumov, 1960). Until a clarifying study of the relationship of these two is published, I prefer to follow Hancock, Drinnan and Harris (1956) in recognizing the two as distinct species.

This hydroid is important in the "white weed" fishery of Britain (Hancock, Drinnan and Harris, 1956).

Reported distribution: Northern Canada to North Carolina; Louisiana.

Sertularia cupressina Linnaeus, 1758
Figure 5F

Sertularia cupressina Linnaeus, 1758, p. 808; Calder, 1970, p. 1531, plate 7, fig. 1; Vervoort, 1972, p. 183.

Thuiaria cupressina Procter, 1933, p. 124; Fraser, 1944, p. 298, fig. 283; Bousfield and Leim, 1960, p. 14.

Collection records: 0518, 0616, 0710, 0714, 0718, 1028, 1130, 1228, 1232, 1428, 1526, 1530, 1612, 1628, 1714, 1812, 1828, 1914, 2220, 2224.

Description: Hydroids mostly fragmentary, largest colony 11.5 cm high. Hydrocaulus monosiphonic with distinct, somewhat irregularly spaced nodes. Internodes moderately long, each having one to three (usually two) branch-bearing apophyses, each apophysis with an axillary hydrotheca; occasional internodes with an additional hydrotheca or a pair of subopposite hydrothecae distally. Perisarc thick and horn-colored in older portions of colony. Branches moderately long, arching upward and outward, dichotomously rebranched, usually given off alternately from opposite sides of hydrocaulus but occasionally arising in opposite or nearly opposite pairs. Branches in all but younger colonies twisted basally, the broad surface being horizontal; internodes having from two to five pairs of hydrothecae, distance between hydrothecae on same side of a given internode 187–245 μ. Hydrothecae subopposite, immersed for much of their length; distal portion curving outward and occasionally somewhat upward. Adcauline wall convex, 268–345 μ adnate, 47–105 μ free; abcauline wall concave, 245–350 μ long. Margin with two distinct teeth; operculum consisting of an adcauline and a larger abcauline valve.

Gonothecae similar in size and morphology to those of *T. argentea*.

Remarks: Specimens of this species may be easily confused with *S. argentea*. Based on the work of Hancock, Drinnan and Harris (1956), *S. cupressina* can be distinguished from *S. argentea* in having (1) branches that arise in one plane, are less regularly rebranched, and more elongated, and (2) hydrothecae that are more deeply immersed and less divergent distally. The hydrothecae of *S. cupressina* are more robust than those of *S. argentea.*

Reported distribution: Labrador to New Jersey.

Sertularia latiuscula Stimpson, 1854
Figure 5G

Sertularia latiuscula Stimpson, 1854, p. 8.

Thuiaria latiuscula Fraser, 1944, p. 303, fig. 289; Brunel, 1962, p. 40; 1970, p. 18.

Collection records: 0718, 1028, 1130, 1220, 1428, 1628, 1714, 1914, 2016, 2114, 2118, 2318.

Description: Colonies largely fragmentary, reaching 8.5 cm high. Hydrocaulus monosiphonic, with several very short internodes at proximal end; divided elsewhere at more or less regular intervals by distinct nodes. Internodes of moderate length, each with one or two branch-bearing apophyses having axillary hydrothecae and up to three pairs of subopposite hydrothecae. Perisarc thick, horn-colored in older portions of colony. Branches moderately long, unbranched or dichotomously subdivided once and given off alternately from opposite sides of hydrocaulus; twisted at base in distal portions of colony and directed upward nearly parallel to hydrocaulus. Distal portion of branch straight or curving gradually outward. Branch internodes each with one to 12 pairs of hydrothecae; distance between adjacent hydrothecae on same side of a given internode 105–304 μ. Hydrothecae subopposite or subalternate, immersed for much of their length except in young colonies and curved outward relatively little, their main axis nearly parallel to that of the branch. Adcauline wall convex, 256–396 μ adnate, 23–117 μ free; abcauline wall more or less convex basally, concave distally, 221–350 μ long. Margin with two teeth; operculum of an adcauline and a larger abcauline valve.

Gonothecae absent.

Remarks: Existing descriptions of this species, and particularly the original description by Stimpson (1854), are rather meagre. As a result, these specimens were identified as *S. latiuscula* only after considerable study. Although the the type specimens could not be located, the hydroids from Cape Cod Bay were compared with material identified as this species from the type locality of Grand Manan Island, New Brunswick (U.S.N.M. No. 29255); specimens from the two areas were virtually identical in morphology. Fraser (1944) was evidently mistaken in describing the operculum of *S. latiuscula* as consisting of an abcauline valve only, because hydroids from both Cape Cod Bay and Grand Manan had a small adcauline in addition to the larger abcauline valve.

Cape Cod Bay hydroids of *S. latiuscula* are readily distinguishable from *S. argentea* and *S. cupressina* in their colony form. Branches in these specimens were either unbranched or dichotomously branched but once, and the branch internodes were generally longer and had a larger number of hydrothecal pairs. Hydroids identified as possibly this species from Georges Bank by A. E. Verrill (U.S.N.M. No. 29253) were more branched, particularly toward the distal end of the colonies, so that some approached the colony form of *S. cupressina.*

Reported distribution: Gulf of St. Lawrence to Virginia.

ADDITIONAL RECORDS

The species of hydroids listed below have been reported from Cape Cod Bay in the literature but were absent in samples from the biotic census. Together with records from the present collection, these bring the number of hydroid species identified from Cape Cod Bay to 33.

Family Tubulariidae

Tubularia couthouyi L. Agassiz, 1862. Fraser, 1944, p. 94, fig. 68.
Tubularia crocea (L. Agassiz, 1862). Fraser, 1944, p. 97, fig. 70.

Family Pandeidae

Leuckartiara octona (Fleming, 1823). Fraser, 1944, p. 58, fig. 29 (as *Perigonimus repens*).

Family Haleciidae

Halecium muricatum (Ellis and Solander, 1786). Fraser, 1944, p. 197, fig. 176.

Family Campanulariidae

Eulaomedea flexuosa (Hincks, 1861). Fraser, 1944, p. 116, fig. 87 (as *Campanularia flexuosa*).
Eulaomedea neglecta (Alder, 1856). Fraser, 1944, p. 126, fig. 98 (as *Campanularia neglecta*).

Family Sertulariidae

Diphasia fallax (Johnston, 1847). Fraser, 1944, p. 242, fig. 227.
Thuiaria lonchitis (Ellis and Solander, 1786). Fraser, 1944, p. 304, fig. 290.

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SUMMARY

1. Twenty-five species of hydroids were identified in samples taken during the Biotic Census of Cape Cod Bay. Of these, only *Corymorpha pendula, Acaulis primarius, Hydractinia echinata, Gonothyraea loveni, Sertularia cupressina,* and

S. latiuscula were particularly common. Eleven of the 25 species were found only once or twice. Bottom type throughout much of the bay is sand or silty-sand, and the general paucity of firm substrates is evidently a primary factor limiting hydroid abundance and diversity in the area.

2. Eleven species are recorded from Cape Cod Bay for the first time. Present records extend the known distribution of *Euphysa farcta* and *Acaulis primarius* southward, and that of *Stylactis arge* and *Lovenella gracilis* northward. As expected, the hydroid fauna of Cape Cod Bay is characteristically boreal in its zoogeographic affinity, and many of the species represented are amphi-Atlantic.

3. *Halecium gracile* Verrill, 1874 is placed in synonymy with *H. halecinum* (Linnaeus, 1758), *Tubularia cristata* McCrady, 1857 is regarded as a synonym of *Ectopleura dumortieri* (van Beneden, 1844), and *Tubularia crassa* Fraser, 1941 is synonymous with *Corymorpha pendula* L. Agassiz, 1862.

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TISSUE CULTURES OF CIRRIPEDS

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The *in vitro* techniques of explantation have been used in two different areas of research. For cell cultures the aim is to to obtain cell outgrowth from an explant and multiplication of the outgrown cells. Continuous cell lines may eventually be obtained. In organ cultures, on the other hand, the goal is to maintain the structural and functional integrity of the explant. Cell outgrowth should be avoided in organ culture, since the escape of cells disorganizes the explant.

Organ cultures have been obtained from malacostracan crustaceans and used in developmental, physiological, and endocrinological studies (Gomot, 1972). Most successful are perhaps the experiments by Berreur-Bonnenfant (1972) on the endocrine activity of the androgen gland in organ cultures of Amphipoda and Isopoda. Cell outgrowth has been reported from explants of decapod crustaceans (Peponnet and Quiot, 1971). Long term cell cultures have been obtained from tissues of the crayfish *Astacus pallipes* and the crab *Pachygrapsus marmoratus* (Vago and Quiot, 1969). Proliferating cells producing large surface spreads were obtained for four months in these cultures. Continous cell lines, however, have not been established from crustaceans.

In Cirripedia neither cell nor organ cultures have been reported. The number of unsolved problems concerning the physiology, development, and endocrinology of cirripeds calls for the development of *in vitro* culture techniques for this group. The present paper describes experiments which aim at establishing techniques and culture media suitable for tissue cultures in cirripeds.

MATERIALS AND METHODS

Materials

Specimens of the acorn barnacle *Balanus amphitrite* were collected on the pilings of the dock at Duke University Marine Laboratory, Beaufort, North Carolina, during the months of April to August 1973. Adult animals in the size range of 10 to 15 mm in basal diameter and preferably with a flat basis, were used. The animals were brushed clean and kept in areated aquaria at 23 ± 1° C and a sea water salinity of 30‰. At least one week of acclimation was allowed between collection and the experiments. During this period of time animals which had been injured during the collecting process could be removed. The animals were fed newly hatched *Artemia* nauplii (Metaframe, San Francisco Bay Brand) every second day. The water was changed weekly and the animals were then cleaned by light brushing. The animals were maintained for months in a good condition as judged by their feeding and molting activity.

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TABLE I

Culture media tested on barnacle tissues. Additions to the barnacle saline of Hoyle and Smith (1963) are listed in percent of the final media, except for Minimum Medium Eagle and Medium 199 where percentages refer to per cent of the concentrations recommended for vertebrate cells.

Medium	Glucose %	Bovine embryo extract %	Yeastolate %	Serum %	Minimum Medium Eagle %	Medium 199 %
I	0	0	0	0	0	0
II	0.1	0	0	0	0	0
III	0.1	1	0	0	0	0
IV	0.1	1	0.1	0	0	0
V	0	1	0.1	0	0	0
VI	0.1	0	0.1	0	0	0
VII	0.1	1	0.1	*Callinectes*, 10	0	0
VIII	0.1	1	0	*Callinectes*, 10	0	0
IX	0.1	1	0	Bovine, 10	0	0
X	0.1	1	0	*Callinectes*, 10 Bovine, 5	0	0
XI	0.1	1	0	*Callinectes*, 10 Bovine, 10	0	0
XII	0.1	0	0	Chicken, 10	0	0
XIII	0.1	0	0	Bovine, 10 Chicken, 5	0	0
XIV	0	1	0.1	0	10	0
XV	0.1	1	0	0	0	10
XVI	0.1	1	0	*Callinectes*, 10	0	10
XVII	0.1	1	0	*Callinectes*, 10 Chicken, 5	0	10
XVIII	0.1	1	0	*Callinectes*, 10 Chicken, 5	0	100

Tissue sampling

Before dissection animals were carefully cleaned and placed for 16 to 20 hours in a hood with the basal membrane pointing towards an utraviolet lamp (30 W) at a distance of 15 cm. On dissection the basal one third of the animal was dipped into 5% sodium hypochlorite for 2 seconds, rinsed in absolute ethanol for 5 seconds, and wiped with sterile cotton. The animal was mounted upside down under a low power microscope and the calcareous basis was carefully removed. Pieces of 1 to 2 mm^3 of mantle tissue with ovarioles and cement glands were sterile dissected out. One of the pieces was immediately fixed in Carnoy (3 ethanol: 1 acetic acid) and the other pieces were used for the cultures. Animals were discarded when the inner mantle membrane was ruptured during the dissection since cultures from these animals were inevitably contaminated.

Culture technique

Pieces of mantle tissue were transferred to Falcon plastic tissue culture flasks (25 cm^2). Each animal gave an amount of tissue sufficient for 2 to 4 flasks each with 4 to 6 explants. The explants were allowed to adhere to the bottom surface of the flask for 30 to 60 seconds before 2 ml of culture medium were applied. About half of the explants remained attached while the others were free floating in the medium. The flasks were immediately gassed with a mixture of 5% CO_2 in air, capped, and incubated at 26° C in darkness. The medium was kept at a pH of 7.3 by gassing with the mixture of CO_2 in air when necessary. Phenol red was used as a pH indicator. The culture medium was changed every fourth day. The cultures were left undisturbed for the first 48 hours, whereafter the cultures were conrolled daily in a Zeiss inverted phase contrast microscope. The amount of cells migrating from the explants, and the quality and multiplication of these cells were studied. Contaminated cultures were discarded. Free floating explants were removed at certain intervals and fixed in Carnoy for histochemical studies. In some cultures explants attached to the plastic surface were removed and fixed to study the origin of the migrated cells.

Culture media

The tested culture media were based upon the barnacle saline of Hoyle and Smyth (1963) : 466 mM NaCl, 8 mM KCl, 20 mM $CaCl_2$, 12 mM $MgCl_2$, 10 mM $NaHCO_3$. The saline without $NaHCO_3$ was autoclaved (30 min at 18 psi). The $NaHCO_3$, prepared in a 5.6% solution and sterilized by filtration (millipore filter, pore diameter 0.22 μ), was added to the saline just before use. The tested culture media were prepared of barnacle saline with one or more additions as shown in Table I. Each medium was tested in 2 to 5 cultures. D-glucose (Sigma) was made up in a 20% solution and autoclaved. Dessicated TC Bovine Embryo Extract EE_{100} (Difco 5396), TC Yeastolate (Difco 5577), and TC Chicken Serum (Difco 5553–72) were resuspended in sterile, distilled and deionized water and stored at –20° C. TC Bovine Serum, ultrafiltrate (Difco 5473–72) was stored at –20° C. TC Minimum Medium Eagle, dried (Difco 0675) and TC Medium 199, dried (Difco 5701) were added directly to the saline in a concentration of 10 or 100 per cent of that recommended for vertebrate cells, and the solutions were immediately sterilized by filtration. Hemolymph of the blue crab, *Callinectes sapidus*, was sampled from the sinus in the merus of the chelipeds in adult, interecdysial (Passano, 1960) animals. Prior to bleeding the crabs were acclimated for 2 to 6 days to sea water of 30‰. The hemolymph was immediately heated to 60° C for 5 minutes to deactivate polyphenol oxidase (Wyatt, 1956). Hemolymph from 20 to 30 crabs was pooled and centrifuged at 31,000 × g for 20 minutes at 4° C in a Sorvall RC2-B centrifuge. The supernatant was filtered through a Millipore filter of pore diameter 0.65 μ with a prefilter, followed by sterile filtration with a prefilter. The filtrate was stored at –20° C. Penicillin (Sigma, Pen-na Benzylpenicillin Sodium Salt) and streptomycin (Sigma, Streptomycin Sulphate) were added to the culture media in concentrations of 100 I.U. per ml just before use.

Histology

The pieces of mantle tissue and cultured explants were fixed in Carnoy for 2 hours, washed twice in absolute ethanol, embedded in Paraplast (Fisher Scientific), and sectioned 5 or 8 μ. The sections were stained routinely with the Mallory-Heidenhain Azan Stain (Koneff, 1938) and with the Feulgen technique

(Pearse, 1968) with Fast green as a counter stain. For histochemical studies the following methods were used: general proteins by Mercuric brom phenol blue after Bonhag (Pearse, 1968); tyrosine containing proteins by the Millon reaction, Baker modification (Pearse, 1968); protein bound SH-groups by the DDD reaction (Barnett and Seligman, 1952) with maleimide block and iodoacetate block (Pearse, 1968) as controls; protein bound SS- and SH-groups by reaction with thioglycollic acid (Pearse, 1968) followed by the DDD reaction (Barnett and Seligman, 1952); RNA and DNA by the Methyl-green-pyronin Y method (Kurnick, 1955) with hydrolysis of RNA by ribonuclease as control; acid mucopolysaccharides and RNA by Toluidine blue O (Pearse, 1968) with ribonuclease as control. Photomicrography was made with a Zeiss Photomicroscope II.

Cultures of other species

Additional cultures were set up with tissues of two other balanids, *B. eburneus* and *B. improvisus*. Specimens of *B. improvisus* (13 to 15 mm in basal diameter) were collected in April 1973 on dead submerged tree trunks at Cherry Branch, Neuse River, North Carolina, and *B. eburneus* (15 to 25 mm in basal diameter) were collected on the laboratory dock in August 1973. The specimens of *B. eburneus* were molt-staged according to the method of Davis, Fyhn and Fyhn (1973) using rami of the sixth pair of cirri. Otherwise the treatment of the animals and the culturing procedure were as described for *B. amphitrite*. Twenty-one cultures were obtained with tissues of *B. eburneus* and 7 cultures of *B. improvisus*.

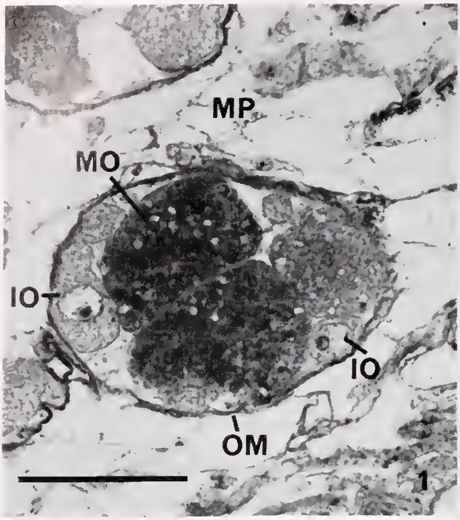
RESULTS

Organ cultures

Quality of culture media was determined by comparing the histology of free floating explants with the histology of uncultured tissue of the same animals (the *in vivo* condition). The explants were cultured for three and seven days. Three different types of ovarian cells were used in the comparison: mature oocytes, immature oocytes, and oogonia.

In the *in vivo* condition (Fig. 1) the mature oocytes of *Balanus amphitrite* were spherical with a diameter of 50 to 60 μ, and the cytoplasm was filled with yolk granules staining bright red with Azan. Immature oocytes were observed in various stages of development having a finely granulated cytoplasm which stained yellow with Azan. The oogonia were seen as small, closely packed cells frequently in mitosis.

The culture media (I-XIX) were divided into four groups (A-D) each with media approximately equal in quality. Medium X did not contain ovarioles in the free floating explants cultured for seven days and this medium was therefore excluded from the comparison. Group **A** consists of four media (III, VI, V, IV) found best for maintaining ovarian cells *in vitro*. In these media no significant difference was found between the histology of uncultured tissue and explants cultured for three days (Fig. 2). On the seventh day of culture the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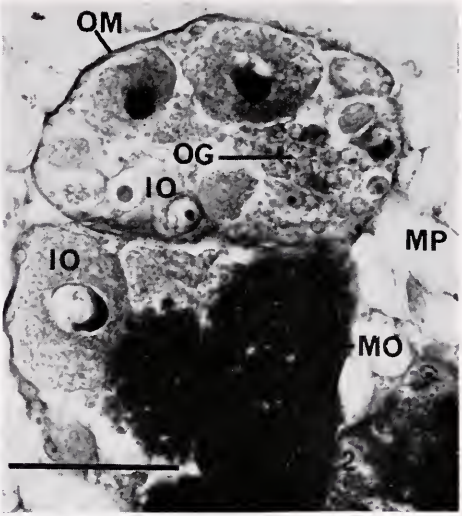
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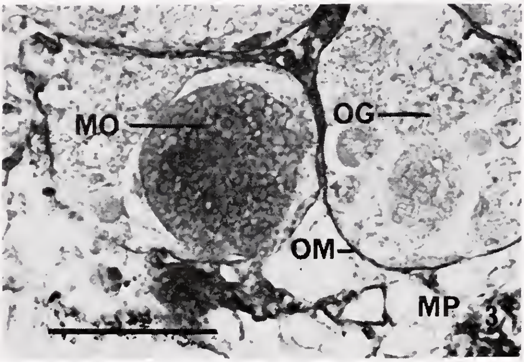

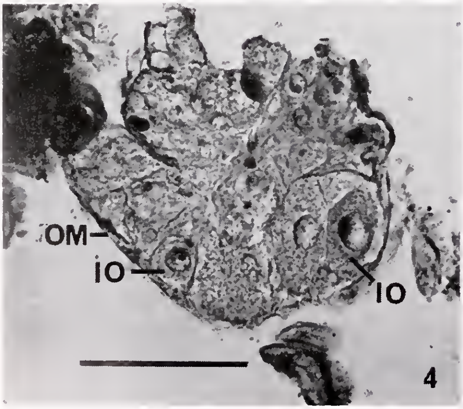

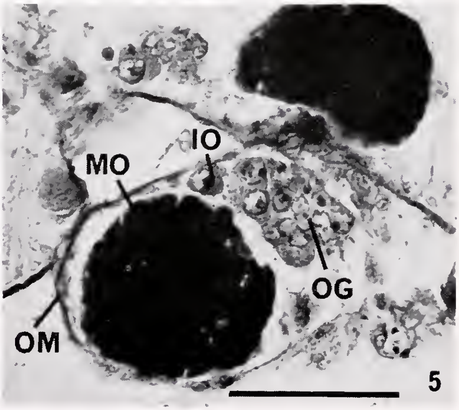

FIGURES 1–3. Ovarioles of *Balanus amphitrite* (Azan stain). Figure 1 shows uncultured tissue (Section 5 μ); Figure 2 shows explant cultured for 3 days (medium VI, section 8 μ); Figure 3 shows explant cultured for 7 days (medium III, section 5 μ); OM—ovariole mem-

mature oocytes were still spherical and the yolk droplets unchanged (Fig. 3). The diameter of the oocytes was normal (medium VI and V), slightly enlarged (medium III) or reduced (medium IV). Small immature oocytes adjacent to the oogonia had a normal appearance. Immature oocytes in later stages of development had a cytoplasm looser in texture than normal. The oogonia seemed normal and were in frequent mitoses. In media of group **B** (VIII, IX, XII, XVIII) the mature oocytes were deformed and the immature oocytes were enlarged already on the third day of culture. The yolk granules were unchanged in number and stainability on the third as well as on the seventh day. No clear mitosis was observed in any of the cultures. In media of group **C** (XI, XIII, XIV, XVII) the oocytes were deformed and a decrease in number of yolk granules was seen on the third day of culture. The stainability of the granules with Azan was reduced in medium XI and XVII, but remained unchanged in medium XIV. After seven days of culture the ovarian cells seemed to be in a poor condition. Still, however, the yolk granules in medium XIV and XIII had a normal appearance. In media of group **D** (I, II, VII, XV, XVI) a degeneration of most of the ovarian cells could be observed already on the third day of culture. On the seventh day the yolk granules of the mature oocytes had disappeared and the nuclei were pycnotic. The immature oocytes and oogonia were partly or completely degenerated in these media.

In some of the media free floating explants were studied histologically later than on the seventh day of culture. In medium VI, the mature oocytes on the 12th day of culture were slightly deformed but with normal yolk granules. The immature oocytes had cytoplasm of loose texture. The oogonia appeared normal, but no mitosis was observed. On the 16th day in this medium most of the yolk granules of the mature oocytes had disintegrated and the cytoplasm of the immature oocytes was partly degenerated. In cultures with media of group **B**, **C** and **D** the degeneration of the ovarian cells observed on the 7th day of culture had progressed further on the 12th and the 16th day.

Parenchymatous cells located between the ovarioles were not observed in mitosis, neither *in vivo* nor *in vitro*. In free floating explants these cells kept their structure and fishnet-like organization with no apparent increase in cell number (Fig. 2). Explants in medium VI had parenchymatous cells of normal appearance after 25 days of culture.

Cement glands in explants cultured in media of group **A** and **B** had normal appearance after three and seven days of culture: The glands had normal size, the cytoplasm was stained yellow by Azan (Fig. 6), and the nucleus contained a high amount of DNA (Fig. 7). In media of group **C** and **D** the cement glands had normal size, but the cytoplasm had a texture looser than normal. The nucelus was enlarged, but the chromatin seemed normal.

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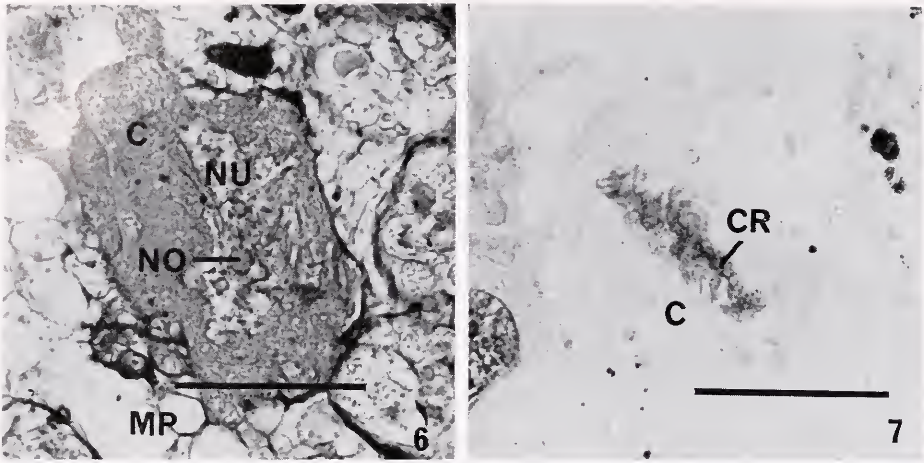
brane, MO—mature oocytes, IO—immature oocyte, OG—oogonia, MP—mantle parenchyma; marker is 50 μ.

FIGURE 4. Explant of *Balanus eburneus* cultured for 3 days (medium III, section 8 μ, Azan stain); OM—ovariole membrane, IO—immature oocyte; marker is 50 μ.

FIGURE 5. Explant of *Balanus improvisus* cultured for 3 days (medium III, section 8 μ, Azan stain); OM—ovariole membrane, MO—mature oocyte, IO—immature oocyte, OG—oogonia; marker is 50 μ.



FIGURES 6–7. Cement glands of *Balanus amphitrite* cultured for 7 days (medium III, section 8 μ). Figure 6, Azan stain; Figure 7, Feulgen reaction and Fast green; C—cytoplasm, NU—nucleus, NO—nucleolus, CR—chromatin, MP—mantle parenchyma; marker is 50 μ.

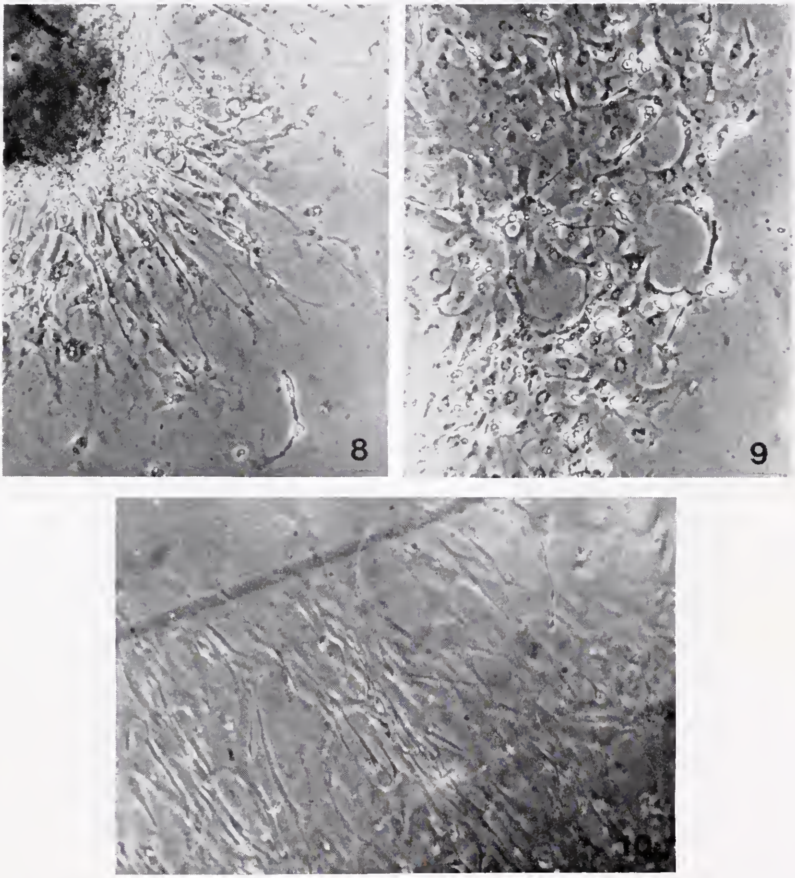
Histochemical tests for proteins, carbohydrates, and nucleic acids were applied to sections of uncultured tissues and of some of the explants. In uncultured material the yolk granules of mature oocytes showed positive reactions for proteins and protein bound SS- and SH-groups, but showed negative reactions for nucleic acids and acid mucopolysaccharides. The cytoplasm of immature oocytes was slightly positive for proteins, strongly positive for RNA (methyl-green-pyronin Y) and was stained dark blue by Toluidine blue. By treatment with ribonuclease the cytoplasm was negative for Toluidine blue, indicating that the blue staining was due to ribose of the RNA. The cytoplasm of cement glands showed negative reactions in the protein tests, but was strongly positive for RNA and stained dark blue with Toluidine blue. The latter test was negative after treatment with ribonuclease.

In explants cultured in media of group **A**, **B** and **C** having mature oocytes with intact yolk granules the histochemical tests showed that the granules kept their normal reactions regardless of number of yolk granules, and of size and shape of the oocytes. When the granules were partly disintegrated (medium XI, XVII and XVI) they showed slight basophilic reactions. Immature oocytes having a normal appearance after three days of culture (media of group **A**) showed normal reactions in the histochemical tests. With decreasing quality of the immature oocytes, the basophilia of the cytoplasm decreased. Cement glands cultured in media of group **A** and **B** showed normal histochemical reactions although the basophilia of the cytoplasm was somewhat lower on the 7th day.

Cultures of mantle tissues of *B. improvisus* and molt staged *B. eburneus* were obtained in various media. Free floating explants were fixed after 3 and 7 days of culture for histological study. The results from these cultures were similar to those obtained for *B. amphitrite* and did not modify the ranking of the culture media for organ cultures. Media of group **A** gave better maintenance of ovarian cells from *B. eburneus* (medium III, Fig. 4) and from *B. improvisus* (medium IV, Fig. 5) than media of group **B** (*B. eburneus:* medium IX, XII, XVIII; *B. improvisus:* medium VIII), group **C** (*B. eburneus:* medium XIII), and group **D** (*B. eburneus:* medium I, XV). No difference was evident between cultures from specimens of *B. eburneus* in interecdysis (11 cultures) and proecdysis (6 cultures).

Cell outgrowth

In each culture 2 to 3 of the explants were attached to the surface of the culture flask. Within the first 48 hours of culture, cells had migrated from most of the attached explants. The migrating cells adhered to the surface. Cells were never observed to migrate from free floating explants, and no cells were seen free in the medium. Cells migrated from explants consisting of both parenchyma and ovarioles, or from explants without ovarioles, but not from explants consisting of ovarioles alone. The migrating cells represented two cell types: epithelial-like



FIGURES 8–10. Cell cultures of *Balanus amphitrite* (phase contrast microscopy). Figure 8 shows epithelial-like cells on the 3rd day of culture (medium III); Figure 9 shows epithelial-like cells on the 5th day of culture (medium III); Figure 10 shows fibroblast-like cells on the 9th day of culture (medium IX).

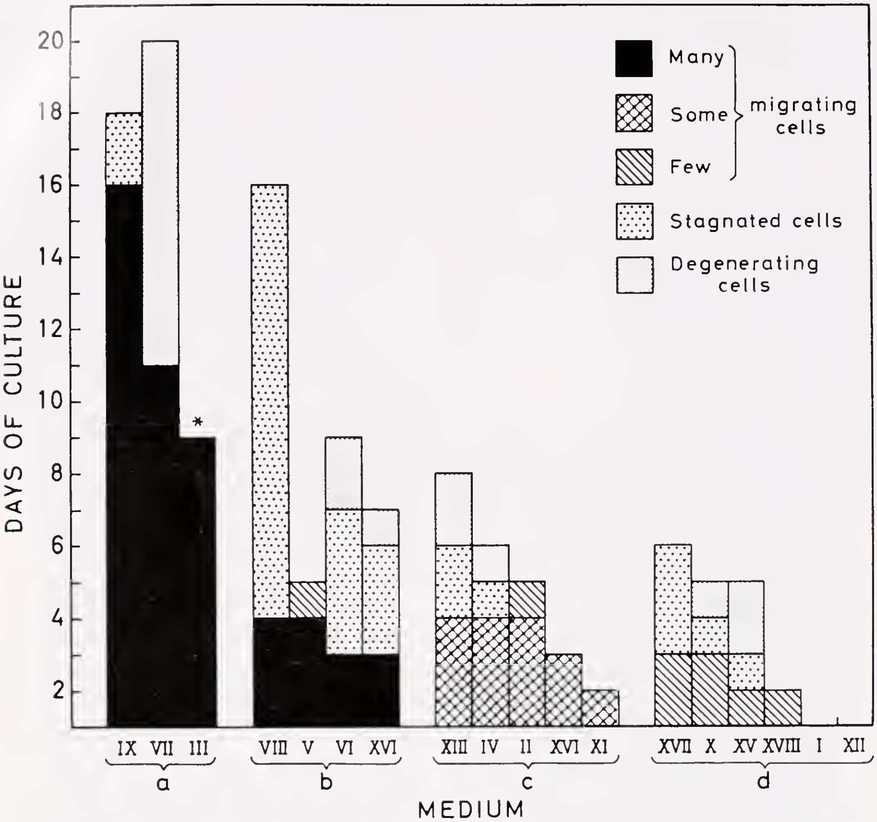
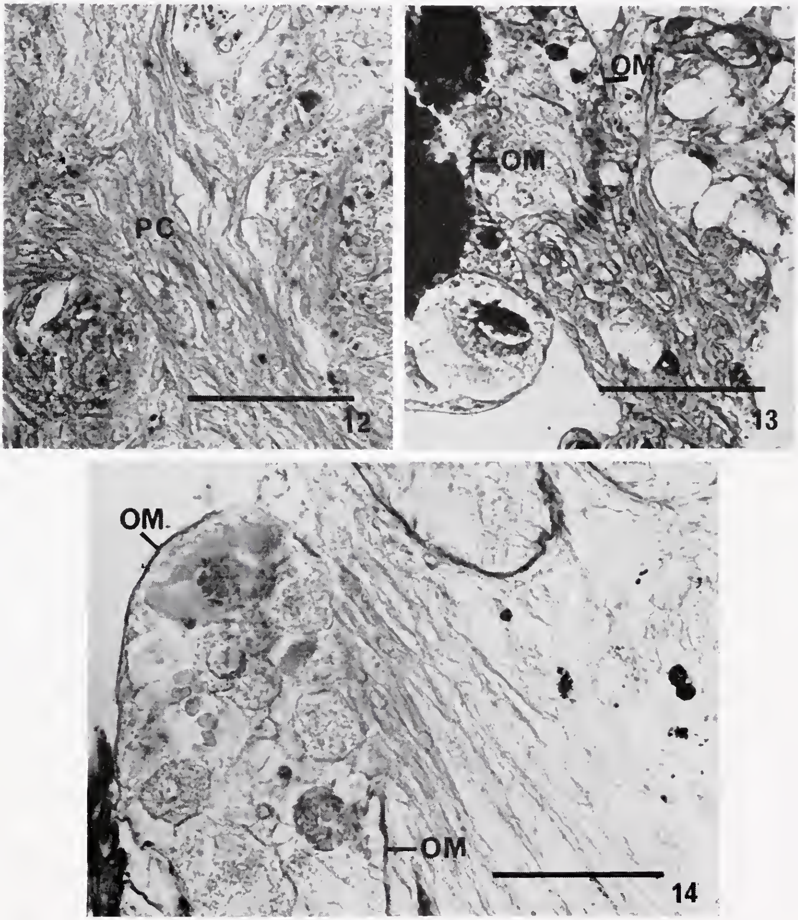


FIGURE 11. Cell outgrowth from explants of mantle tissue of *Balanus amphitrite*. The culture media are ranked according to the amount, quality and duration of migrating cells obtained in each medium. The most successful culture in each medium is illustrated.

cells which were polygonal with granules surrounding the nucleus (Fig. 8) and fibroblast-like cells which were more or less elongated and spindle shaped (Fig. 10). The epithelial-like cells were the first to migrate from the explant. Migrating cells generally had hyaline cytoplasm and mitoses were frequently observed in good cultures. After 2 to 16 days, depending upon the culture medium, the migration of cells and the frequency of cell divisions tapered off. The cells then increased in size and vacuoles and granules were observed in the cytoplasm. This stagnated phase could last for 10 days when a degeneration or cell death became evident: the vacuoles and granules in the cytoplasm became dominant, the cells became fringed and their attachment to the plastic surface gradually loosened. This phase of obvious degeneration varied from ½ to 9 days and terminated by the disappearance of the outgrown cells.

The number, quality, and life span of the migrating cells clearly depended upon the culture medium (Fig. 11). The media in this figure are ranked according to the success of the cell outgrowth obtained in each medium and are divided into four groups (**a**-**d**) consisting of media approximately equal in quality. In the three media comprising group **a** (medium IX, VII, and III) numerous cells migrated from the explants. Epithelial-like cells were most abundant during the first 7 to 9 days (Figs. 8 and 9). From then on the mitotic activity in the cultures increased and fibroblast-like cells became the far most abundant cell type (Fig. 10). The migrating cells had good appearance with hyaline cytoplasm and high mitotic activity up to the 17th day of culture. The cultures in medium IX and VII showed a sharp transition from an active proliferating phase to stagnation or degeneration, and the outgrown cells disappeared after 18 to 20 days of culture (Fig. 11). The culture in medium III, which is illustrated in Figure 11,



FIGURES 12–14. Explants being source of migrating cells (Sections 8 μ, Azan stain). Figure 12 shows parallel cells in the parenchyma (PC) (*Balanus eburneus,* 7th day of culture, medium IX); Figure 13 shows ovariole membrane (OM) partly broken (*Balanus amphitrite,* 5th day of culture, medium V); Figure 14 shows ovariole membrane (OM) partly broken (*Balanus eburneus,* 7th day of culture, medium IX); marker is 50 μ.

was terminated on the 10th day. Three other cultures with this medium (plus five cultures of *B. eburneus*) were terminated after 6 to 7 days while still in a proliferating phase with a heavy outgrowth of cells. Probably, therefore, proliferation may be obtained in medium III for a longer period than the nine days illustrated. Group **b** is composed of four media in which numerous cells migrated from the explants for 3 to 4 days. The cells had normal appearance with some mitotic activity, but soon disappeared or went into a stagnated phase. In group **c** five media are included in which some cells migrated from the explants during the first 2 to 5 days of culture. The cells generally had normal appearance, but no mitoses were observed. In media of group **d** only a very sparse migration of cells or none at all (medium I and XII) was obtained. The cells were irregular and fringed with heavily granulated and vacuolized cytoplasm, and no mitoses were observed.

Seven cultures with attached explants were obtained from mantle tissues of *B. improvisus* and 21 of molt staged *B. eburneus*. In the media of group **a** heavy cell migration was obtained in cultures of *B. eburneus* (8 cultures) and of *B. improvisus* (2 cultures) for 7 days when the cultures were terminated. In media of group **b** (VIII and VI) three cultures of *B. improvisus* showed some migrating cells for 3 to 4 days; in media of group **c** (XIII, IV, and XVI) two cultures of each species showed some migrating cells for 3 to 4 days; in media of group **d** (X, XV, XVIII, I and XII) eleven cultures of *B. eburneus* did not result in any cell migration. No differences were evident between cultures from specimens of *B. eburneus* in interecydsis (14 cultures) and proecdysis (7 cultures).

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The histology of attached explants was studied in cultures of *B. amphitrite* and *B. eburneus* to investigate the origin of the outgrown cells. Explants attached to the surface of the culture flasks (medium V and IX) and surrounded by migrating cells were scraped off, fixed and studied. In the *in vivo* condition the ovarioles were bounded by a continuous cellular membrane, and the parenchyma between the ovarioles had a fish-net appearance (Fig. 1). In free floating explants these conditions remained unchanged (Figs. 2–6). In attached explants the cells in the parenchyma were orientated parallel to each other (Fig. 12) while the cellular membrane of the ovariole usually was kept intact. In two cases the ovariole membrane was partly broken and an outgrowth of ovarian cells seemed probable (Figs. 13 and 14).

DISCUSSION

The saline of Hoyle and Smyth (1963) has been successfully used in physiological experiments on barnacles (Hoyle and Smyth, 1963; McLaughlin and Hinke, 1966; Gwilliam, 1968) suggesting that the saline electrolytically is compatible with the requirements of cirriped tissues. The results of cultures with pure saline as a medium in the present study showed that cell maintenance for more than a few days as well as cell migration and multiplication depend upon exogenous sources of nuritional support besides the compatible ionic environment. D-glucose in a concentration of 0.1% did not improve the saline as a culture medium (medium II versus I) and neither did it improve a more complex medium (medium IV versus V). In cultures of gonads of the amphipod *Orchestia gamarella* Berreur-Bonnenfant (1962) found a culture medium of sea water with 0.5% glucose (plus agar as a solidifier) to be inferior to the same medium enriched with chicken embryo extract. Gonad survival was 4 days and 35 to 40 days in the respective media. Vago and Quiot (1969) reported 0.1% glucose to be included in a complex medium for cell cultures of embryonic cells of the crayfish *Astacus pallipes*. The effect of glucose on the cell cultures, however, was not investigated.

Bovine embryo extract and yeastolate in concentrations of 1% and 0.1% respectively, were found to be very good additions to the media for organ cultures as well as for cell outgrowth. Bovine embryo extract and yeastolate are complex additions and probably served as source of vitamins, amino acids, proteins, and carbohydrates. Higher concentrations of bovine embryo extract as well as addition of other tissue extracts, could further improve the media for barnacle tissue cultures.

The addition of serum had a different effect on cell maintenance and on cell migration. The four best media for organ cultures (group **A**) are without serum. For cell outgrowth the addition of 10% bovine serum or *Callinectes* hemolymph had a positive effect (medium IX versus medium III, medium VII versus IV). Chicken serum, however, seemed to have a negative effect on cell migration (medium XII versus II). Bovine serum was a more favorable addition for cell outgrowth than *Callinectes* hemolymph (medium IX versus VIII). The *Callinectes* hemolymph was pooled from animals in interecdysis. Hemolymph of crabs in other molt stages may be a more potent growth stimulator. The positive effect of serum on barnacle cell migration and proliferation is in accordance with the assumed effects of serum in control of cell multiplication in vertebrate cell cultures (Temin, Pierson and Dulak, 1972). Grace (1968) found the growth of cells in insect cell cultures to increase with hemolymph concentrations up to a certain limit when inhibitory effects appeared. For barnacle cell outgrowth more than 10% of serum had a negative effect (medium XI and X versus medium IX and VII, Fig. 11). In decapod crustaceans Eagle minimum medium and medium 199 have been successfully used in cell cultures (Vago and Quiot, 1969) and in organ cultures (Oyama and Kamemoto, 1970; Skinner and Beattie, 1971). For the barnacle cultures, however, the addition of these media did not improve the culture media.

In organ cultures loss of cells from explants is a difficulty often encountered but did not represent a problem in the present cultures. The proteid yolk granules showed high capacity for maintenance. Even under conditions when the oocytes and other ovariole cells were in an advanced state of degeneration, the yolk granules had a normal appearance. Mature oocytes were maintained for at least seven days in cultures with the four best media. In organ cultures of crab ovaries mature oocytes did not survive (Oyama and Kamemoto, 1970). In the barnacle cultures the mitotic activity of oogonia was maintained and the small immature oocytes adjacent to the oogonia seemed normal. Larger immature oocytes, however, showed an incipient degeneration on the seventh day suggesting that even the best media could not support the previtellogenetic development required for yolk deposition. Immature oocytes in a late stage of development did neither survive in crab ovary cultures (Oyama and Kamemoto, 1970) while small immature oocytes and oogonia were maintained.

Extensive cell outgrowth with high mitotic activity was obtained from attached explants for 18 days in medium IX. At the onset of stagnation the outgrown cells formed continuous sheets surrounding each explant. Subculturing at an earlier stage may prolong the cultures in this medium. In medium VII heavy cell proliferation was obtained for 12 to 13 days. This medium was the poorest one tested for maintenance of ovarian cells in organ cultures. Parenchymatous cells, however, had a normal appearance for at least seven days. Medium VII thus seems to support cell migration and proliferation, but cannot support the maintenance of specialized ovarian cells in culture. Medium III seems to be suitable for organ cultures as well as for cell outgrowth. It was the second best medium for cell maintenance and one of the best for cell outgrowth. Tissue cultures as obtained by the present technique should allow investigations with physiological, endocrinological, and pathological aspects of barnacle cells *in vitro*.

Ovarian tissue is generally a fruitful source of migrating cells in primary cultures. The origin of migrating cells from such complex organs as the ovary is, however, difficult to determine (Vaughn, 1971). In the barnacle cultures the explants included unspecialized parenchymatous cells and/or ovarian cells in various stages of development and specialization. Migrating cells were possibly of non-ovarian origin since ovarioles in explants being donors of migrating cells usually were kept intact, the parenchymatous cells in donor explants had a paraellel orientation (Fig. 12), and cell migration was never observed from isolated ovarioles. In two cases, however, the ovariole membrane was partly broken and an outgrowth of ovarian cells seemed probable (Figs. 13 and 14). In insect ovarian cultures Jones and Cunningham (1960) assumed the boundary membrane of the follicular cells to be a barrier for the penetration of these cells into the cellular field, and Grace (1958) found no cell migration from naked ovaries. Stanley and Vaughn (1968) circumvented the problem by using minced ovaries. Dispersion of the cells in barnacle explants at the start of the cultures may increase the ovarian part of the cell donation. With the general high proliferation potential of ovarian cells and the frequent cell divisions obtained in germinal cells in the present cultures, this may increase the lifespan and growth capacity of barnacle cells *in vitro*.

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SUMMARY

1. Various components were tested as nutrients in media for barnacle tissue cultures. Bovine embryo extract and yeastolate were favorable additions for both cell outgrowth and organ cultures. Glucose did not improve the media. Bovine

serum and *Callinectes* hemolymph had a negative effect. Mixed sera in concentrations higher than 10% were negative for both types of cultures. Addition of Minimum Medium Eagle and Medium 199 did not improve the barnacle culture media.

2. In organ cultures mantle parenchyma were obtained for 25 days, ovarioles and cement glands for at least 7 days with no change in structure and organization. Mature oocytes and young immature oocytes were maintained for at least 7 days, and oogonia kept a high mitotic activity. Immature oocytes in later stages of development were not maintained.

3. Extensive cell outgrowth forming large surface spreads and with cells showing high mitotic activity were obtained from attached explants for 18 days. The initial cell migration consisted of epithelial-like cells, later, fibroblast-like cells were most abundant. A predominantly non-ovarian origin of the migrating cells seemed probable. It is suggested that the ovarian part of cell donation may be increased by early dispersion of the cells in the explants.

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HEAT EFFECTS ON A MARINE SNAIL

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The ability of intertidal prosobranch molluscs to survive high temperatures is related to their position in the intertidal zone and to their geographical distribution (Gowanloch and Hayes, 1926; Broekhuysen, 1940; Gunter, 1957; Southward, 1958; Fraenkel, 1961, 1966, 1968; Sandison, 1967; Newell, 1970; Markel, 1971). The high lethal temperature of *Littorina littorea* at Woods Hole, Massachusetts was 1–2° C lower in the winter than in the summer; also small differences exist between individual snails of the same species and these differences correlate with the relative locations of the individuals in the intertidal zone (Fraenkel, 1968). The heat tolerance of isolated tissues and enzymes correlates with the resistance of the intact animals and the heat resistance of tissues of many species are identical in summer and winter (Dzhamusova, 1960; Ushakov, 1964; Zhirmunsky, 1967). Intertidal prosobranchs are exposed to considerable temperature fluctuations during a tidal cycle, and often to great temperature variations seasonally. Thus *Littorina littorea* at Woods Hole is alternately frozen in the winter (Kanwisher, 1955, 1959, 1966) and heated by the hot summer sun. Even tropical snails may experience large temperature variations diurnally with tidal exposure and seasonally at the extremes of their ranges. For example, Lewis (1963) found tissue temperature differences in exposed specimens of *Nerita tesselata* in Barbados ranging from 6.0° C above to 14.0° C below ambient sea surface temperatures, and inshore water temperatures as low as 14.6° C and air temperatures of 11.2° C in February were found in the Florida Keys in an area near the 28.8° C isotherm for the maximum average annual sea surface temperature (Stephenson and Stephenson, 1950).

Mechanisms in prosobranchs compensatory for conditions of seasonal temperature fluctuations have been found in several species. For example, *Littorina littorea* can survive low temperatures better in the winter than in the summer (Kanwisher, 1966; Sömme, 1967). Segal (1965) reported that both high-level and low-level intertidal populations of the limpet, *Acmaea limatula,* had a faster heart rate in the winter at any given temperature than did summer animals. Effects of temperature on oxygen consumption also correlate with intertidal and geographic distribution (Newell, 1970; Sandison, 1967).

Ohsawa and Tsukuda (1956) noted a shift toward lower optimal temperatures for the extruding response of winter specimens of the periwinkle, *Nodilittorina granularis.* Except for *N. granularis,* few data exist dealing with the behavioral aspects of acclimation in prosobranchs.

Marine snails, when heated, typically enter a reversible state of heat coma several degrees below the temperature from which they fail to recover. This coma consists of detachment of the foot from the substratum and immobility, usually with the foot extended (Fraenkel, 1968; Sandison, 1967). The present study tests the effect of acclimation upon the temperature of heat coma in the common Atlantic Coast periwinkle, *Littorina littorea* which occurs primarily between high neap tides and low water spring tides. This study also compares thermal limits for foot reflexes and muscle contractions in cold and warm adapted specimens of *Littorina littorea*. These behavioral changes are correlated with changes that occur in the spontaneous activity of the central nervous system.

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MATERIALS AND METHODS

Specimens of *Littorina littorea*, obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, were acclimated to different temperatures in ten gallon aquaria half-filled with artificial sea water (Instant Ocean) in rooms maintained at 5° C, 15° C, and 25° C. The snails were maintained on a constant 12 hour light cycle and were not fed during the period of acclimation. For measurements of heat coma, snails were removed from one of the constant temperature rooms and placed in a seawater bath of about 2.3 liters capacity at the temperature at which they had been acclimated. The temperature was then raised in stepwise fashion; the bath temperature was held constant for five minutes, and then was raised by 1° C; held for five minutes and then raised again. Temperature was controlled at each step to within less than 0.5° C by means of a knife-blade type heater controlled by a mercury thermostat and a "minitrol" relay. Uniformity of temperature throughout the bath was achieved by vigorously bubbling air through the water.

In order to ascertain how quickly a snail reaches temperature equilibrium with the bath, a thermistor probe was inserted through a small hole drilled through the shell of a *Littorina* of 1.8 cc volume and sealed to the shell. The snail was then placed in a bath of 6° C. As soon as the body of the animal reached temperature equilibrium with the bath, the snail was transferred to another bath at 21° C. In three trials the time required for the snail to reach equilibrium with the bath at 21° C was 57–60 seconds, giving an approximate rate of temperature rise of about 0.25° C per second.

Lethal temperatures were measured on snails collected in February and acclimated for from three weeks to several months at 0.5° C, 5° C, and 25° C. The snails were placed directly into sea water previously heated to a given temperature and kept there for one hour (Fraenkel, 1960). The bath was aerated throughout the experiment. At the end of one hour the animals were removed and returned to 15° C sea water to permit recovery. Those snails that subsequently reattached to the container and crawled up the side and out of the water were considered to have recovered. Snails which behaved in this way following subjection to high temperatures were kept for three weeks in aquaria and appeared to be normal at the end of this time.

The hypothesis of muscle failure as an explanation of heat coma was tested by a series of length-tension meaurements at different constant temperatures using muscle preparations from 5° C and 25° C acclimated snails. The columellar muscle was chosen for test purposes because it is the muscle that pulls the foot into the shell and is involved in the heat coma phenomenon through its failure to cause withdrawal of the foot while the animal is in heat coma.

To obtain a muscle preparation, the shell was cracked with a nutcracker to expose the body and to leave the attachment of the columellar muscle to the columella intact. The visceral mass was excised, the head slit open, and the organs of the head and foot removed, including all the major ganglia of the central nervous system. The shell remnant (columella) was then placed in a clamp attached to a Palmer stand, the muscle was attached to a photodiode mechano-electrical transducer by a 2 inch length of silk thread which was tied to a pin inserted through the operculum, and the preparation was immersed in sea water in a battery jar of 2.3 liters capacity and aeration was maintained. Sea water was an adequate medium for maintaining active muscle preparations for at least a week at 10° C. Electric stimuli were administered as monophasic square pulses of 15 volts, 20 msec pulse duration at 4 pulses per second from a Grass stimulator and isolation unit via chlorided silver electrodes which were inserted into the middle of the muscle mass, about 2 mm apart. The electrodes were insulated by three coats of spar varnish, with about one millimeter of the ends exposed. Isometric tension was amplified by a direct current amplifier and recorded by a Sanborn recorder, model 320. Temperature of the bath was held constant by means of a 125-watt knifeblade type heater and a mercury contact thermometer. Temperatures below room temperature were maintained by packing dry ice around the bath.

Contraction characteristics of the 5° C-acclimated and 25° C-acclimated snails were compared at several test temperatures over the range 5° C to 40° C. Standard length was taken to be the length at which the tetanic tension was greatest at each test temperature.

Expression of tension development as a function of cross-sectional area of the columellar muscle was complicated by the fact that the dimensions of the muscle cannot practically be determined, as this muscle becomes intermeshed with fibers from other muscles in a most intricate manner upon its entry into the foot. To obtain an approximation of the dimensions of the muscle, it was assumed that the thickness of the contracting muscle was uniform throughout its length, from the point of attachment on the columella to that on the operculum. The standard length of muscles from six snails was determined by progressively stretching the muscle, stimulating and recording tension development. As soon as peak tension was passed, the muscle was returned to the length at which peak tension was developed. At this length all excess flesh of the head and foot was excised, leaving a muscle strip of uniform diameter similar to that near the point of attachment to the columella. This strip was then weighed, and in all cases was found to be approximately 50% of the eviscerated, deganglionated muscle preparation. The weight of the columellar muscle was, therefore, taken to be 50% of the untrimmed muscle preparation in all animals.

Knowing the weight and the length of a muscle, the tension development in grams per square centimeter cross-sectional area was determined by the equations: 1) $P = \frac{F}{A}$ which equals $P = \frac{F}{v/l}$, rearrangement gives $P = \frac{Fl}{v}$; 2) also, $P = \frac{m}{v}$ and $v = \frac{m}{\rho}$. Substituting for v in equation 1 gives $P = \frac{Fl\rho}{m}$; assuming ρ is approximately equal to 1, then $P_0 = \frac{F_0 l_0}{m}$ where P = tension, A = cross-sectional area of the muscle, F = force, m = mass, ρ = density, l = length, v = volume, P_0 = tension developed at standard length, and l_0 = standard length of the muscle.

Spontaneous activity of the central nervous sytem of *Littorina littorea* was measured using a suction electrode of inside diameter 0.2 mm, into which the severed end of a nerve tract leading from a ganglion was drawn. Spontaneous action potentials were amplified by means of a Tektronix 122 low level preamplifier, time constant 0.2 seconds, and were displayed on a Tektronix 502 dual beam oscilloscope. Records were obtained photographically at a film speed of 100 mm per second.

A nerve preparation consisted of the intact circumeosophageal nerve ring containing the cerebral, pleural, and pedal ganglia from a snail previously acclimated to either 5° C or 25° C. This preparation was obtained from the snail by opening the head, removing the buccal mass, salivary glands, and gut, thereby exposing the ganglia. The nerve ring was then removed and placed in a small lucite chamber, the bottom of which was covered with paraffin wax. The preparation was held in position by a small tungsten staple which was slipped over the connectives and inserted in the paraffin. The preparation was bathed by a constant flow of sea water through the chamber from a large lucite reservoir maintained at constant temperature. Aeration and mixing in the reservoir were achieved by bubbling air through the sea water. All equipment used in these experiments except the oscilloscope and kymograph camera were enclosed in a Faraday cage. Temperature was monitored by a thermistor probe placed in the nerve chamber close to the ganglia. During most experiments the starting temperature was 5° C. This temperature was maintained for 15 minutes before recording the nervous activity. Kerkut and Taylor (1958) found that 3 to 5 minutes equilibration of the slug isolated ganglia was sufficient to obtain steady rates of spontaneous activity. This was confirmed in the present study and a 5 minute equilibration period was allowed at each experimental temperature before spontaneous activity was recorded. During the course of an experiment the temperature in the lower ranges was raised by 5° C increments. Preliminary experiments revealed a difference in the temperature of cessation of spontaneous activity in the 5° C-acclimated and the 25° C-acclimated ganglia; therefore, the temperature was raised by 1° C increments above 25° C for the 5° C-acclimated ganglia and above 30° C for the 25° C-acclimated ganglia, in order to establish the temperature of cessation of activity for each.

Initially, measurements were made of the spontaneous action potential frequency recorded from the largest of the pedal nerves from the pedal ganglion, but the gross spike frequency was very high, with the spikes tending to occur in bursts. It was subsequently found that the spontaneous activity of the tentacular nerve leading from the cerebral ganglion was of lower and more regular spike frequency. As cessation of activity in the two ganglia occurred at the same high temperatures, the right tentacular nerve was chosen for all subsequent experiments. A total of 14 experiments was performed in which 7 nerve preparations were from 5° C acclimated snails and 7 were from 25° C acclimated animals.

Results

Heat coma

A marine snail immersed in a seawater bath behaves in a characteristic manner when exposed to rising temperatures. This consists of ever increasing activity (crawling up the sides of the container), which begins to diminish at higher temperatures, finally resulting in complete cessation of motility. The snail's response to temperature change is quite rapid; an active snail placed directly at high temperatures becomes inactive within a few seconds. At the temperature of complete immobility the tentacles may still wave about. With further increase in temperature the foot begins to curl back away from the substratum, usually beginning at the anterior end. This process continues until the animal falls to the bottom of the container. An animal which may have been attached to the bottom of the vessel also releases its attachment. If the elevated temperature is maintained, the snail lies on the bottom of the vessel, usually with the foot fully extended, showing few, if any, spontaneous movements. In all experiments in this study the criterion for heat coma was taken to be the temperature at which the animal relinquished its hold upon the substratum. In this state the snail would usually withdraw in response to prodding, but reflex activity was abolished if the temperature was raised 3 to 4° C higher. If the temperature was then lowered, reflex activity returned within 10 to 15 seconds, and reattachment and apparently normal behavior returned in most individuals within 5 to 15 minutes.

The effect of acclimation upon the temperature of development of heat coma in *Littorina littorea* is shown in Table I. Some specimens of *Littorina* obtained from Woods Hole were acclimated at 5° C, 15° C, or 25° C for 14 days, others for 50–55 days. Attempts to acclimate *L. littorea* at 30° C failed because the snails invariably died after five to six days at this temperature. One group of *L. littorea* were acclimated for 14 days at 0.5° C. The heat coma temperatures increased from 30.5° C for 0.5° C acclimation to 38.8° C for 25° C acclimation.

Table I

The effect of acclimation temperature upon temperature of heat coma in Littorina littorea.

Temperature of acclimation	Acclimation time (days)	Number of snails	Mean temperature of heat coma *E.S.E.	t-test for significance of difference between means of 5° C- and 25° C-acclimated snails	Date snails were collected
0.5° C	14	10	30.5° C ± 0.81		Feb. 1968
5° C	14	10	32.5° C ± 0.53	$P < 0.001$	Apr. 1967
15° C	14	10	35.0° C ± 0.38		Apr. 1967
25° C	14	10	38.8° C ± 0.42		Apr. 1967
5° C	54	10	31.6° C ± 0.62	$P < 0.001$	Apr. 1967
15° C	55	9	35.9° C ± 0.70		Apr. 1967
25° C	50	10	38.9° C ± 0.41		Apr. 1967

* Estimated standard error.

TABLE II

Heat coma temperatures of Littorina littorea *taken directly from the sea at Woods Hole, Massachusetts.*

Date of collection	Number of individuals	Mean temperature of heat coma *E.S.E.	Temperature of sea water at start of experiment	t-test for significance of difference between means of December snails and July 18 snails
July 18, 19, 1966	10	37.6° C ± 0.16	22.5° C	$P < .05$
Dec. 20, 22, 1965	5	32.6° C ± 0.51	22.0° C	

* Estimated standard error of the mean.

Variance analysis of the data shows no significant difference between the heat coma temperatures of *L. littorea* acclimated for the two times of 14 and 50–55 days. Thus acclimation was complete by two weeks.

The reversibility of the shift in heat coma temperature was shown by an experiment in which ten *L. littorea* were acclimated at 25° C for two weeks, then returned to 5° C for two weeks. The mean heat coma temperature of the snails after two weeks acclimation at 5° C was 32.6° C. This agrees well with the heat coma temperature of snails that had remained at 5° C (Table I).

In Table II heat coma temperatures for summer and winter specimens of *L. littorea* are compared. At the time of collection of the summer animals in July, the ambient sea surface temperature at Woods Hole was 22.0° C to 22.5° C. The winter snails were shipped by air from Woods Hole to Chicago in December. The mean heat coma temperature of the winter snails is significantly below that of the summer animals; this provides evidence that acclimatization occurs seasonally in the sea.

Lethal temperatures

Fraenkel (1960, 1968) found for *Littorina littorea* that animals tested in March died after one hour at 39° C while summer animals died after the same time at 40–41° C. I found that snails acclimated to 5° C showed a survival rate of 7 out of 10 at 40° C and 1/10 at 41° C, whereas animals acclimated to 25° C showed a 5/10 survival at 41° C. Snails acclimated at 0.5° C when heated at 40° C for one hour had a survival rate of 7 out of 10 in each case. This shows that acclimation in the laboratory shifts the lethal temperature of snails by about the same amount as seasonal acclimation in the field as measured by Fraenkel (1968).

For any species of animal there are many "lethal temperatures" according to the measured time to death (Orr, 1955a). Thirty degrees Centigrade, constantly maintained, was a lethal temperature for *L. littorea* over the course of four to five days. Attempts to acclimate the snails slowly to high temperatures by keeping them at 25° C for varying lengths of time prior to placing them in the 30° C room consistently failed. As *L. littorea* can survive for months at 25° C, the lowest lethal temperature for this species in the laboratory lies between 25° C and 30° C.

Muscle measurements

The heat coma could be explained by failure of muscles to perform at high temperatures, by the inability of the nervous system to initiate muscular activity, or by both occurring simultaneously. As the temperature of heat coma has been demonstrated to shift approximately 8.5° C between the acclimation temperatures 0.5° C and 25° C in *Littorina littorea,* a change in the ability of muscles to respond to stimuli should occur as a result of acclimation if heat coma is caused by failure of muscles.

In both the 5° C-acclimated and 25° C-acclimated columellar muscles, the developed tension showed an approximately linear decline over the experimental range 5° C to 40° C (Figure 1): there was no significant difference in maximum tension developed by 5° C- and 25° C-acclimated muscles at any given temperature ($P > .05$). This decline in muscle tension with increase in temperature could be the result of an increase in the rate of the relaxation processes and/or increased rate of decay of the active state. No measurable muscle contraction could be elicited from either warm or cold acclimated muscles at 40° C. Table III shows that both the time to peak tension and time to one-half relaxation decreased with increase in temperature.

A difference was noted in the relaxation rates of the 5° C- and 25° C-acclimated muscles when both were tested at 10° C, but not at 25° C (Table III and Fig. 2).

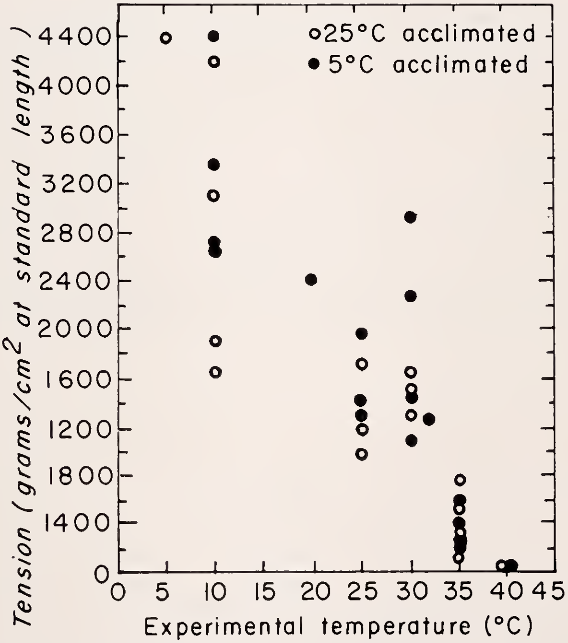

FIGURE 1. Maximum isometric tetanic tension of the columellar muscle of *Littorina littorea* acclimated at 5° C and 25° C measured at different experimental temperatures; open circles represent 25° C-acclimated, closed circles represent 5° C-acclimated muscles.

Table III

Comparison of the time to maximum tetanic contraction and to one-half relaxation of the columellar muscle of 5° C-acclimated and 25° C-acclimated L. littorea *at 10° C and at 25° C.*

Number of muscle preparations	Acclimation temperature	Mean time to peak tension *E.S.E.	Mean time to ½ relaxation	Test temperature	t test for significance of difference between means in relaxation data at 10° C
4	5° C	37.9 ± 2.6	35.5 ± 5.1	10° C	$P < 0.05$
4	25° C	27.1 ± 7.0	16.5 ± 4.2	10° C	
5	5° C	4.2 ± 0.4	4.3 ± 0.5	25° C	
3	25° C	4.7 ± 0.4	4.3 ± 0.7	25° C	

* Estimated standard error of the mean.

The time to maximum contraction was not significantly different, but the relaxation phase was significantly longer in the 5° C-acclimated muscles than in the 25° C-acclimated muscles.

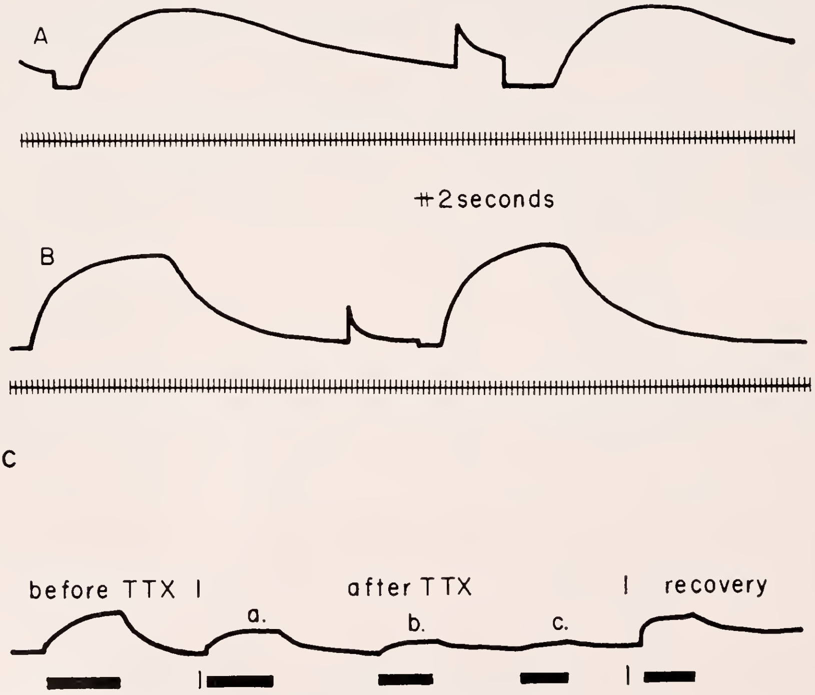

Figure 2. Contractions of *L. littorea* columellar muscle at 10° C showing the difference in time to one-half relaxation in (A) 5° C-acclimated muscle and (B) 25° C-acclimated muscles; (C) the effect of 1×10^{-6} gm/ml tetrodotoxin on isometric contraction of the columellar muscle of *L. littorea;* time after application of TTX; in (a) 12 minutes (b) 27 minutes, and (c) 37 minutes. Black bars indicate duration of stimulus.

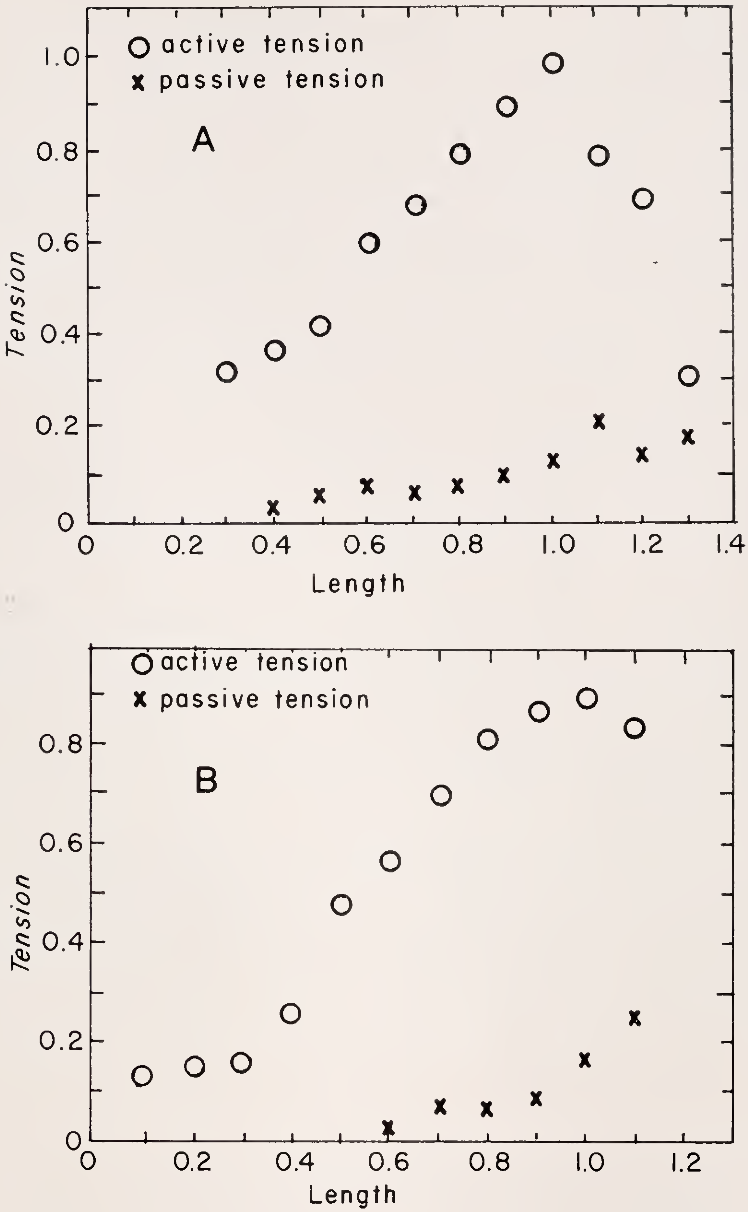

FIGURE 3. (A) Mean of three length-tension measurements of 5° C-acclimated *L. littorea* columellar muscles tested at 10° C. Open circle represents active tension, and X represents passive tension. (B) shows the mean of four length-tension measurements of 25° C-acclimated *L. littorea* columellar muscles tested at 10° C. Open circle represents active tension, and X represents passive tension.

Passive and active length-tension curves are presented in Figure 3 for measurements taken at 10° C. These were superimposable for muscles from 5° C-acclimated (Fig. 3A) and 25° C-acclimated (Fig. 3B) snails at 10° C and at all other temperatures of measurement. It is therefore unlikely that the difference in relaxation time in the 5° C- and 25° C-acclimated muscles at 10° C is caused by a change in the elastic elements of the muscle as a result of acclimation.

Whether or not acclimation temperature has any affect on the ability of the columellar muscle to contract after exposure of the snail to extreme temperatures was tested as follows. Specimens of *L. littorea* were acclimated at different temperatures for at least nineteen days. The intact animals were then placed in a seawater bath previously heated to a given high temperature and were kept at this temperature for one hour. At the end of an hour the snails were removed from the bath and the contractions of the columellar muscles were recorded.

The results of this experiment show that there is at most only a difference of 1° C in the heat resistance of the muscles of the 25° C-acclimated *L. littorea* compared to those of the 5° C-acclimated snails. Muscle response in snails acclimated at 5° C did not fail (10/10 responding) after being heated at 43° C for one hour, but failed (0/10) after one hour at 44° C. In muscles from animals acclimated at 25° C, there was no failure after one hour at either 43° C (10/10) or 44° (10/10), but failure of response was complete (0/10) after one hour at 45° C.

A possible role of the central nervous system in influencing muscle heat resistance was found in experiments in which isometric twitch responses of the columellar muscle of *intact* 5° C- and 25° C-acclimated *L. littorea* were compared at different high temperatures, starting at room temperature. Both the 5° C- and the 25° C-acclimated muscles failed to respond to electrical stimuli at 43° C. This was at least 3° C *higher* than the temperature of muscle failure in deganglionated preparations.

These experiments show that acclimation temperature of the snail does not change the heat resistance of the columellar muscle by more than 1° C to 2° C.

In the preceding experiments the muscle was stimulated electrically. There is histological evidence that a nerve plexus exists in the foot musculature of gastropods (Bullock and Horridge, 1965): this plexus may also be present in the columellar muscle. Several attempts were made by use of methylene blue and Bodian's stain to find a nerve plexus in the foot and columellar muscle but no positively identifiable nervous elements could be found other than large nerve tracts. The possibility exists, however, that electrical stimulation of the columellar muscle occurred indirectly *via* a plexus, or other peripheral nerves, rather than directly.

Failure of the muscle to contract at high temperatures might have been the result of a temperature effect upon the neuromyal junction or on the peripheral nerve rather than upon the muscle. In an attempt to ascertain whether or not the muscle was being directly stimulated, the following nerve-blocking drugs were applied: xylocaine, procaine, d-tubocurarine, hexamethonium, probanthine, tricaine-methane-sulfonate, and tetrodotoxin (TTX). The concentration of drug used in most instances was 1×10^{-4} g/ml except TTX which was used in a concentration of 1×10^{-5} g/ml. In order to enhance penetration of the drugs, the muscles were usually trimmed until a strip of 2 millimeters thickness remained. In some experiments the preparation was exposed to the drug for several hours in order to ensure complete penetration of the drug. The preparations were kept at 10° C to prevent possible deterioration of the muscle; preliminary experiments had shown that these preparations could be kept in the cold for many hours without noticeable effect upon muscle contraction.

None of the drugs except tetrodotoxin (Fig. 2C) abolished the columellar muscle contraction. The abolition of contraction by tetrodotoxin tends to support the hypothesis that columellar muscle contraction is mediated by nerves, but since tetrodotoxin is known to block sodium conductance in some muscles as well as in nerves (Narahashi, Moore, and Scott, 1964) the question still remains whether the muscles ceased to contract because of nerve block or because the muscle was directly affected. Twarog (1967) found that tetrodotoxin in high concentrations had no effect upon spike amplitude or contraction of *Mytilus* anterior byssus retractor muscle (ABRM) bundles when the muscle cells were stimulated directly. Moreover, direct application of tetrodotoxin to the isolated ganglia of *L. littorea* blocked all spontaneous nervous activity within five minutes; activity subsequently returned upon washing the ganglia with sea water. From the absence of effects of the acetylcholine-antagonizing drugs, it appears that if stimulus of the columellar muscle is indirect, then the excitatory transmitter is possibly not acetylcholine.

Several deganglionated muscle preparations of *L. littorea* were held at 5° C for varying periods of time (up to several days) in an attempt to ascertain whether or not there might be a decline in the ability of the muscle to contract which might be attributable to nerve degeneration. The amplitude and other characteristics of contraction of the deganglionated muscle preparation held in the cold for six days were indistinguishable from those of a freshly dissected preparation. Several other preparations in which the pedal ganglia were left intact were held at 5° C for 15 days. At the end of this time the muscle preparation did not respond to electrical stimuli, although some spontaneous action potentials were recorded from nerves from pedal ganglia. In view of the fact that muscle contraction was unaffected in the deganglionated muscle after six days at 5° C and that neurones in the pedal ganglia survived after two weeks in the cold, it seems possible that the peripheral nerves may survive as long as the muscle fibers themselves, particularly if there are ganglion cells associated with the nerves. The failure of the muscle to contract after fifteen days in the cold could as well be attributed to loss of viability of the muscle cells as to nerve degeneration.

Spontaneous nervous activity in isolated ganglia

The previous section indicates that heat coma and its modification by acclimation are not the result of temperature effects on muscle. The hypothesis that heat coma depends upon effects in the central nervous system was tested by measurements of electrical activity in central ganglia at different temperatures.

Figure 4 gives the normalized mean spike frequency in a tentacular nerve at different temperatures from fourteen experiments. Figure 4 shows that spike frequency in both the 5° C-acclimated and 25° C-acclimated ganglia increases with increase in temperature up to about 20° C, and declines rapidly above 25° C.

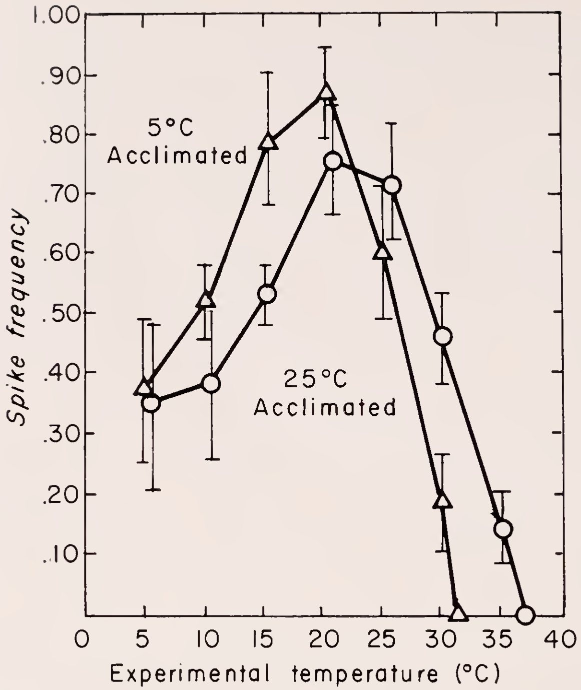

FIGURE 4. The effect of acclimation temperature upon spontaneous activity of isolated ganglion from *L. littorea:* open triangle represents 5° C-acclimated, open circle represents 25° C-acclimated. Range marks indicate standard error of the mean. Frequency is plotted as the ratio of spike activity at a given temperature to the maximum activity exhibited by the preparation over the experimental range. Each point is the mean of 7 experiments.

There is no statistical difference between the frequency of the 5° C-acclimated and 25° C-acclimated ganglia up to 25° C. However, at 30° C the spike frequency of the 5° C-acclimated ganglia is approximately half that of the 25° C-acclimated ganglia. The mean temperature of complete (but reversible) cessation of spontaneous activity for the 5° C-acclimated snails was 31.4 ± 0.5° C, while that of the 25° C-acclimated snails was 37.1 ± 0.8° C. The t test indicates a difference between these means at the 0.005 level of significance. Lack of hysteresis in the temperature effect upon spontaneous activity of the ganglia was shown in that the gross spike frequency at 18° C during cooling after heating to over 31° C was similar to that at 20° C during the rising phase of the temperature experiment.

In a few cases a large repeating unit could be followed, and analysis of these records shows that the increase in gross spike frequency with increase in temperature was the result of both an increase in the rate of firing of the repeating unit and the addition of other units. A plot of the activity of the repeating unit in two 5° C-acclimated ganglia and two 25° C-acclimated ganglia is shown in Fig. 5. There appeared to be no discernible difference between the activity of the repeating units resulting from acclimation except in the temperature of cessation of activity.

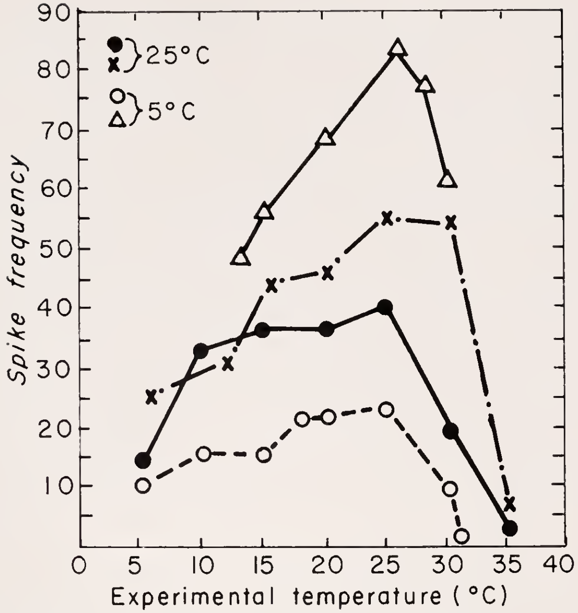

FIGURE 5. Comparison of the frequency of firing of 4 repeating units recorded from the tentacular nerve from two 5° C-acclimated snails and two 25° C-acclimated snails.

DISCUSSION

Littorina littorea is capable of coping with both long-term and short-term changes in environmental temperature. In the present investigation this capability is expressed by a shift in the temperature of heat coma as a result of laboratory acclimation to different temperatures.

Fraenkel (1968) found that *Littorina littorea* apparently has little ability to adapt seasonally in regard to lethal temperature, and a shift of only about 1° C was found for the same species as a result of temperature acclimation in the laboratory in the present study. It is difficult to imagine any adaptive significance in such a small shift in lethal temperature. However, *L. littorea* spends much of its time out of water and is exposed to a much greater range of temperatures than are organisms which show seasonal shifts in lethal temperatures, *e.g.*, fish (Prosser, 1973). The ecological significance of any given "lethal temperature" for a species depends upon whether or not the animal may be exposed to this temperature long enough to be killed or injured to the point where it might fall easy prey to some predator or disease, or suffer damage to the reproductive tissues. It is quite likely that *L. littorea* may survive body temperatures of over 30° C for a period of several hours on hot summer days. Maximum snail body temperatures recorded in the field are usually lower than the maximum tolerated in laboratory tests (Markel, 1971).

It is reasonable to suppose that in intertidal snails the lethal temperature(s) might be set at the genetic limit for the species, and if this limit were sufficiently high in the snail's environment, selective pressures would be exerted upon adaptations that would allow the animal to cope with short-term sublethal temperature stress. A shift in the heat coma threshold would be an example of such an adaptation. The temperature of heat coma found for *L. littorea* was approximately 10° C lower than the lethal temperature. Similar range between coma and lethal temperatures was observed for *L. littorea* from Wales (Sandison, 1967) and for *Monodonta lineata* from the Mediterranean (Micallef, 1968). Coma temperature was more subject to change by acclimation than lethal temperature, hence lethality may involve defects in more and different physiological systems than does coma.

The possibility that heat coma in *L. littorea* may be caused by a direct effect of temperature upon the columellar muscle has been ruled out by the series of experiments in which muscles continue to contract at temperatures above lethal and coma levels and in which the maximum tetanic tension developed by muscles from 5° C- and 25° C-acclimated snails was shown to be not significantly different over the range 5° C to 40° C. These data support the results of Dzhamusova (1960), who found that deganglionated foot muscle preparations of *L. littorea* failed to respond to electrical stimuli after approximately 2 to 3 minutes at 40° C.

The data of this paper are in general agreement with the results of Dzhamusova (1967), Ushakov (1964), and Zhirmunsky (1967), who found that the heat resistance of molluscan muscles was constant within a species regardless of the thermal history of the individuals tested. In two species of *Littorina* Dzhamusova found identical heat resistance in populations taken from 10° C to 12° C waters in the Sea of Okhotsk and from 20° C to 23° C waters in the Sea of Japan.

The measurements of tension development of deganglionated muscle of *L. littorea* agree with the results of Cambridge, Holgate and Sharp (1959) who found a decrease in isometric contractions in *Mytilus* anterior byssus retractor muscle with increase in temperature but these measurements differ from those of Benthe (1954) who found that isotonic twitch contractions of the isolated foot of 3° C-, 12° C-, and 21° C-acclimated *Limnaea stagnalis* increased in amplitude over the experimental range 5° C to 20° C. However, it is difficult to see how Benthe could make valid comparisons of twitch amplitude since his measurements were not based upon tension developed per cross-sectional area of muscle.

Benthe (1954) found that the presence of central ganglia apparently imparted greater heat resistance of *L. limnaea* foot muscle than was exhibited by deganglionated preparations. This observation was supported by a number of experiments in the present study. In intact specimens of 5° C- and 25° C-acclimated *Littorina littorea* isometric twitch responses of the columellar muscle failed at 43° C, a temperature at least 3° C higher than that at which deganglionated muscle preparations failed to respond. Increased heat resistance conferred upon the muscle by central ganglia may explain in part the differences in results obtained by Dzhamusova (1960), who found the "maximum heat of resistance" of deganglionated muscle of *L. littorea* to be about 34° C and those of Fraenkel (1968), who heated intact snails of the same species for one hour and found a maximum temperature of 43° C to 44° C for the opercular reflex.

In the length-tension and contraction experiments it was not known whether the columellar muscle was responding directly to electrical stimuli or indirectly *via* nerves. Attempts to discover the mode of muscle stimulation by means of drug experiments were inconclusive, but a number of experiments reported in the literature have shown that at high temperatures the neuromyal junction ceases to function at lower temperatures than either the muscle or the nerve, as in skate (Battle, 1926) and frog (Orr, 1955b; Jensen, 1972). It is possible that if stimulation of the columellar muscle of *L. littorea* occurred indirectly *via* motor nerves, failure of the muscle to contract at high temperatures may have been the result of failure of the neuromyal junction.

The heat coma phenomenon shows a good correlation with the temperature of cessation of spontaneous activity of the central nervous system as measured *in vitro*. The mean heat coma temperature for *L. littorea* acclimated at 5° C is 32.5 ± 0.5° C and the mean temperature of cessation of spontaneous activity of the isolated ganglia of snails acclimated at the same temperature is 31.4 ± 0.6° C; the heat coma temperature for snails acclimated at 25° C is 38.9 ± 0.4° C, while the mean temperature of cessation of CNS activity is 37.1 ± 0.8° C. It seems reasonable to infer that there is a causal relationship between the two phenomena in that heat coma is the visible expression of cessation of spontaneous nervous activity. It may be concluded that as in other poikilotherms [crayfish, teleosts (Prosser, 1973)] the nervous system of *Littorina* is most vulnerable to thermal extremes.

This work represents part of a dissertation submitted to the University of Chicago in partial fulfillment of the requirements for the Ph.D. degree, granted in December, 1969. I am deeply grateful to Drs. C. L. Prosser and G. S. Fraenkel for their advice and encouragement and to Dr. H. B. Steinbach for his counsel and support. This research was financed by a NASA fellowship and by NSF grant BMS-01587 to C. L. Prosser.

SUMMARY

Littorina littorea enters a reversible state of heat coma at temperatures below the lethal temperature of the species. The threshold of heat coma can be shifted as much as 8.5° C by acclimation; a linear relation exists between heat coma temperature and acclimation temperature over the experimental range.

Deganglionated columellar muscle preparations of 5° C and 25° C acclimated *Littorina littorea* showed an approximately linear decline in tetanic tension when tested from 5° C to 40° C. There was no statistically significant difference in the amplitude of muscle contraction or of tension-length curves of 5° C- and 25° C-acclimated muscles at any experimental temperature; this excludes failure of muscle contraction as the cause of heat coma. Speed of contraction was similar and relaxation slower in muscles from 5° C-acclimated snails than in muscles from 25° C-acclimated snails when measured at 10° C but not at 25° C.

Attempts to ascertain by the use of drugs and nerve degeneration experiments whether muscle contraction was elicited by direct or indirect stimulation were inconclusive, although the effects of TTX were consistent with the hypothesis that stimulation was by nerves.

Lethal temperature in *L. littorea* shows a shift of only about 1° C to 2° C as a result of temperature acclimation. The ability of the columellar muscle to

contract following exposure of the snail to extreme temperatures for one hour was similarly shifted only 1° C to 2° C by acclimation.

Measurement of spontaneous activity of the central nervous system of *L. littorea* showed good agreement between temperature of cessation of spontaneous activity and temperature of heat coma in 5° C- and 25° C-acclimated snails. Analysis of nervous activity showed that increase in activity with rising temperature was based upon both a greater frequency of firing of single units and activation of new units. Single unit frequency appeared to show no acclimation effect except in the temperature of cessation of activity.

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ADAPTIVE FEATURES OF GUT STRUCTURE AND DIGESTIVE PHYSIOLOGY IN THE TERRESTRIAL ISOPOD *PHILOSCIA MUSCORUM* (SCOPOLI) 1763.

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The alimentary canal of isopod crustaceans is comparable to that of most other Arthropoda in that three basic regions can be recognized. These are the foregut, derived from the ectodermal stomodaeum and consisting of an oesophagus and proventriculus, the endodermal midgut, and an ectodermal hindgut derived from the proctodaeum (Goodrich, 1939). The midgut, though, is unusual in that it is virtually restricted to four or six simple caeca which arise at the junction of the fore- and hindgut. Controversy over whether any other part of the alimentary canal is of endodermal origin, and therefore to be regarded as midgut, has been resolved by Holdich (1973) who concludes from a comprehensive review of the literature that the discontinuity between the chitinous intimas of the fore- and hindgut described by Goodrich (1939), underlain in some species by a band of endoderm two to three cells wide, is the only remnant of a true midgut in isopods, other than the caeca.

The terrestrial isopods show further modifications of the alimentary canal in that the hindgut is subdivided into an anterior somewhat expanded region, which bears along its length a dorsal typhlosole, a median papillate region, a muscular sphincter, and a short posterior rectum (Sutton, 1972).

The histology and ultrastructure of the isopod gut is known from a considerable number of studies, summarized in Jones, Babbage and King (1969), Schmitz and Schultz (1969), Holdich and Ratcliffe (1970), Clifford and Witkus (1971) and Alikhan (1972). The caeca have received particular attention and most authors agree that they are the source of endogenous digestive enzymes which act on the food within the caecal lumen, the proventriculus, or the hindgut. The caeca are also concerned with absorption of digested food and in some instances are believed to carry on digestion intracellularly.

Little is known, though, of the functional significance of the reduction of the midgut to four or six caeca and, in terrestrial species, of the subdivision of the hindgut and the development of a typhlosole. It would seem likely, however, that the latter modifications are related in some way to the terrestrial habit, probably as adaptations to the type of food available. The terrestrial isopods have a large proportion of cellulose in their diet, feeding on moist vegetable litter and often supplementing this by consuming their own faeces (Wieser, 1966; Sutton, 1972). They show strong preferences for partly decomposed litter rich in the microorganisms which effect its decomposition (Edwards, 1974; Hassall, 1975a) and their gut modifications may well be adaptive features facilitating efficient utilization of this food, possibly by allowing the isopods to make extensive use of the cellulose-degrading properties of the litter microflora. Endogenous cellulases are not common in animals and herbivores are often dependent on microorganisms for cellulose digestion (Buchner, 1965). In insects these are usually symbiotic inhabitants of the gut (Lasker, 1959) but in isopods, where only a few species have been examined for cellulases, the situation is less clear. Ray and Julian (1952) and Ray (1959a) found no evidence of a microflora in the gut of the marine wood-borer *Limnoria lignorum* and concluded that the cellulases present were secreted by the cells of the gut caeca. This conclusion was also reached by Hartenstein (1964) for the terrestrial *Oniscus asellus,* but no firm evidence for actual secretion from the caecal cells was presented for either species.

The present study has been undertaken to assess the functional significance of the various modifications in mid- and hindgut structure seen in terrestrial isopods, to establish the general pattern of their digestive physiology and to ascertain the origin of the cellulases acting in their gut. The species selected for the study was *Philoscia muscorum* (Scopoli) 1763, an isopod known to be a macrodecomposer in British dune grassland ecosystems and whose diet, feeding mechanisms and foregut functional morphology have already been described (Hassall, 1975b).

Materials and Methods

Philoscia muscorum was collected from a dune grassland at Spurn Head, East Yorkshire and maintained under semi-natural conditions in large fiberglass tanks containing sand, turf and leaf litter from the natural habitat.

Specimens selected for study were starved for five days in 15 cm diameter plastic pots, on 2mm mesh nylon gauze suspended 2 cm above a moist pad of plaster of Paris. Faeces fell through the gauze as they were produced and could not be ingested. This procedure cleared the gut of remnants of previous meals, encouraged subsequent ingestion of test meals and facilitated section cutting by removing ligneous and siliceous particles derived from the natural diet.

The structure of the mid- and hindgut and the course of digestion were studied by histological, histochemical and substrate film methods, using alimentary canals removed either directly from the starved animals or at intervals after the latter had been observed to ingest test meals of boiled potato or well decayed leaf litter.

Histological methods

Whole animals, and intact alimentary systems removed by dissection, were fixed for twenty four hours in Bouin's fluid. Penetration of fixative into the whole animals was facilitated by removing the lateral regions of the pereionites and pleonites. Serial sections were stained by Ehrlich's haematoxylin and eosin, Mallory's triple stain, Feulgen's reaction for D.N.A., or the periodic acid-Schiff reaction. The progressive digestion of starch in potato-fed specimens was followed in whole mounts and sections by staining with Lugol's iodine or periodic acid-Schiff.

Histochemical methods

Specimens were immobilized by cooling in the freezing compartment of a refrigerator. They were then flooded with cold saline [0.34 M sodium chloride as used by Schmitz and Schultz (1969) for *Armadillidium*] and the gut rapidly dissected out. Serial sections were obtained by fixing for twelve hours at 1° C in 10% formalin buffered to pH 7.0, dehydrating in graded cold acetones, clearing in xylene and impregnating *in vacuo* in paraffin melting at 45° C (Jennings, 1962). The sections were examined for enzymic activity using: a) the indoxyl acetate method for non-specific esterase (Holt, 1958) either alone or with various activators and inhibitors (Pearse, 1961); b) the L-leucyl β naphthylamide hydrochloride method for arylamidases (Burstone and Folk, 1956); and c) the naphthyl AS-BI phosphate methods for acid and alkaline phosphatases (Burstone, 1958).

Enzymic activity was also studied by applying the above methods to intact alimentary canals, removed from immobilized starved and fed individuals, fixed by immersion for twelve hours in absolute acetone at 1° C.

Controls for the histochemical investigations consisted of heat inactivated sections, incubation of normal sections in media lacking the specific substrate and incubation in the normal media of sections of appropriate mammalian tissues.

The techniques for acid and alkaline phosphatases indicated some possible sites of absorption. Confirmation of these was sought by using the iron saccharate method of Yonge (1926), feeding starved individuals on boiled potato mixed with saccharated ferrous carbonate. These were fixed at progressive intervals in equal parts of Bouin's fixative and 5% ammonium sulfide in 95% alcohol, and examined for absorbed iron after treating sections with 10% aqueous potassium ferrocyanide followed by 0.1 N hydrochloric acid. Controls consisted of starved individuals fed on potato only, to detect endogenous iron; and of intact alimentary canals removed from starved individuals, filled by injection with saccharated ferrous carbonate and immediately fixed. This demonstrated diffusion artifacts arising during fixation and subsequent procedures.

Substrate film methods

The occurrence and distribution of cellulases were studied using the substrate film method devised by Sumner (1968) in which the tissue or section to be examined is placed beneath a thin film of carboxymethyl cellulose ("Cellofas B 10", I.C.I. Ltd., England). Any cellulases present hydrolyze the film adjacent to their position in the specimen, the hydrolyzed areas subsequently remaining clear when the film is stained with 0.05% toluidine blue. Diffusion of cellulases was reduced by prior fixation of materials in formaldehyde-calcium (Baker, 1960) for one hour at 4° C.

The materials tested were intact alimentary canals, and frozen sections 15 μm thick through various regions of the canal, from individuals fixed after starvation and after meals of either the natural food of decomposing leaf litter or boiled potato. Samples of boiled potato, the natural food and of the faeces were also tested.

Controls for the substrate film tests consisted of heat inactivated specimens, and squashes of the digestive gland of *Helix aspersa* fixed and treated in the same manner as the other materials.

Observations and Results

The alimentary canal in *Philoscia muscorum* (Fig. 1) has the form characteristic of terrestrial isopods, comprising an oesophagus and proventriculus (foregut), four tapering caeca (midgut) which arise from a small common caecal chamber situated ventrally at the junction of the proventriculus and hindgut, and a hindgut subdivided into four distinct regions.

The mouthparts, feeding mechanism and functional morphology of the foregut have been described elsewhere (Hassall, 1975b). Briefly, decomposing leaf litter is fragmented by the mandibles, pushed into the mouth by the other mouthparts and passed down the oesophagus into the proventriculus. The proventriculus presses the food, squeezing out liquids and channelling them ventrally into the midgut caeca while solids are directed into the anterior chamber of the hindgut where they may remain for up to seven days. The walls of the proventriculus bear internal lamellae and when the organ is empty it can be moved by its extrinsic musculature in such a way that secretions from the caeca can be directed

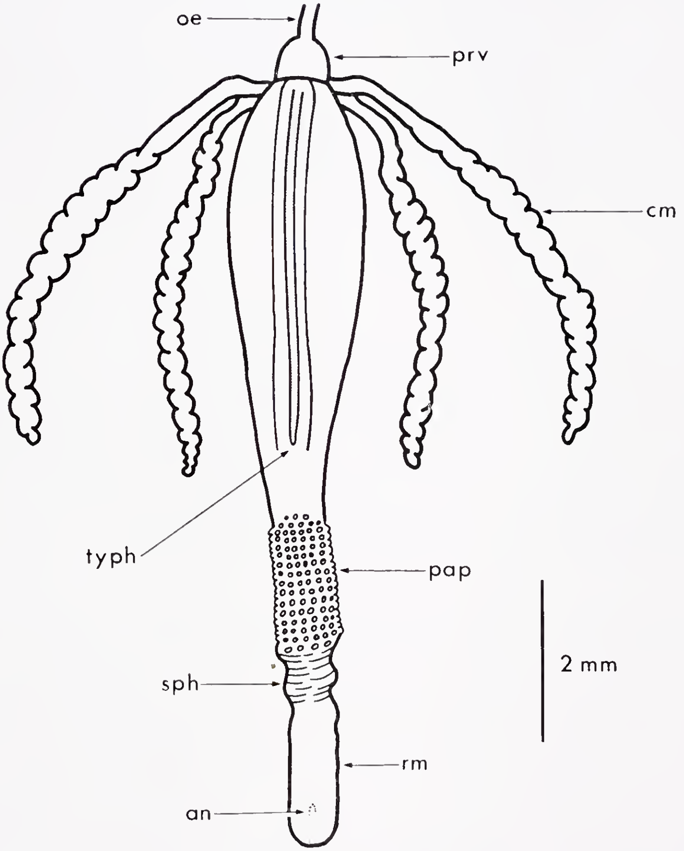


FIGURE 1. Alimentary canal of *Philoscia muscorum* from the dorsal aspect. The origins of the caeca from the common ventral chamber beneath the proventriculus are not shown: an represents position of postero-ventral anal slit; cm, caecum; oe, oesophagus; pap, papillate region of hindgut; prv, proventriculus; rm, rectum; sph, sphincter; typh, typhlosole.

upwards and into the ends of the two channels formed from the upturned lateral projections of the typhlosole which runs along the inner dorsal surface of the anterior portion of the hindgut.

The caeca (midgut)

In adult *P. muscorum* the caeca (Figs. 1 and 2) are 4.0–5.0 mm in length and 0.5–0.8 mm in diameter. They curve upwards and backwards from their origin below the proventriculus and lie freely in the body cavity. Externally they appear as spiral structures due to the presence of thin sheets of muscle which run obliquely around them. The muscles are not differentiated into circular and longitudinal components, but their oblique path presumably compensates for this in that it allows them to change both the length and diameter of the caeca and so draw in, or expel, food materials and digestive secretions.

The principal component of the caecal wall is a monolayered endothelium composed of cells ranging in size and shape from small pyramidal cells, 20–30 μm tall, through various intermediate types to broad columnar ones reaching 70 μm in height and 30 μm in width (Fig. 2). The cells have a striated border 0.5–0.1 μm in depth, are usually binucleate, rarely mono- or trinucleate, with prominent nucleoli and granular basophilic cytoplasm whose basophila increases with the size of the cell. The cytoplasm contains variable amounts of organically bound iron which can be demonstrated after unmasking with ammonium sulfide, when it gives a deep blue color on treatment of sections with potassium ferrocyanide and hydrochloric acid. The amount of iron present varies somewhat between similarly sized cells but there is a clear relationship between it and cell size, in that smaller cells contain only a few granules in their basal regions while larger ones are loaded with iron almost up to their distal borders (Fig. 2).

The caecal cells contain esterases which, like the iron, increase in both amount and distribution within the cytoplasm as the cells increase in size. Small pyramidal cells show either no reaction for esterases or only a thin distal band of activity, but as they grow the esterase zone deepens until the mature full sized cell shows an intense reaction through most of its cytoplasm (Fig. 3). The esterase reaction is thermolabile, being destroyed by holding sections at 90° C for five minutes, while the reaction for iron is thermostable, so there is no possibility that the reaction for esterases is an artifact resulting from visualization of intracellular iron by the ferro- and ferricyanide components of the esterase incubation medium. Further, the iron deposits form in the basal regions of the cells and spread distally as the cells grow, while esterase formation follows the reverse path.

Standard biochemical practice recognizes A-, B- and C-type esterases. The intensity of the reaction was not diminished by pre-incubation of sections in 10^{-5} M E-600 (diethyl-*p*-nitrophenyl phosphate), indicating that B-type esterases such as cholinesterases and lipases are not present in the caecal secretions and that the enzymes responsible for the reaction are probably A- or C-type esterases, or both. The results of including specific inhibitors or activators in the incubation medium after pre-incubation in E-600 suggested that, in fact, both types are present (Table I). The reaction was completely inhibited by 10^{-5} M silver nitrate (as are all A- and C-esterases) but activated by 10^{-3} M cysteine which is a characteristic of A-esterases. These, though, are inhibited by 10^{-4} M sodium *p*-chloromercuri-

TABLE I

Characterization of the E-600 resistant caecal esterase reaction in P. muscorum†

Treatment	Effect	Inference
10^{-2} M $AgNO_3$	complete inhibition	A- or C-esterases present
10^{-3} M cysteine	activation	A-esterases present
10^{-4} M PCMB*	activation	C-esterases present
10^{-3} M $Pb(NO_3)_2$	no inhibition	C-esterases present
10^{-2} M βPPA**	no inhibition	A-esterases present

† following the classification of Pearse (1961).
* sodium *p*-chloromercuribenzoate.
** β-phenylpropionic acid.

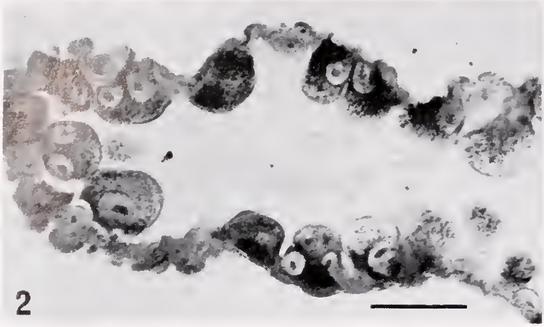
benzoate while the caecal esterases were activated by this compound, a characteristic of C-esterases. Further, the caecal reaction was not inhibited by either 10^{-3} M lead nitrate, which causes 50% inhibition of A-esterases, or 10^{-2} M β-phenylpropionic acid which inhibits C-esterases.

The caecal cells also give a slight positive reaction to the Burstone and Folk method for arylamidases. This, unlike the esterase activity, occurs uniformly throughout the cytoplasm of cells of all sizes and does not increase as the cells grow. They show similar slight but uniformly distributed reactions to Burstone's methods for acid phosphatase (incubation pH 5.2) and alkaline phosphatase (pH 8.3).

The hindgut

The hindgut in *P. muscorum* is the longest part of the alimentary canal (Fig. 1), running almost the entire length of the body and being 9–10 mm long and 1–1.5 mm broad anteriorly in adult specimens. It consists of an outer muscular layer, made up of outer longitudinal and inner circular components, a monolayered epithelium and a thin periodic acid-Schiff positive chitinous intima closely applied to the distal epithelial surface. Variations in the relative thicknesses of the muscular layer, the degree of folding of the epithelium and the shape and disposition of the epithelial cells allow four distinct regions to be recognized, morphologically and histologically. These are termed here the typhlosole, externally papillate, and sphincter regions, and the rectum.

Typhlosole region. The anterior half of the hindgut is characterized by a dorsal typhlosole which runs from its anterior end to a point about 0.8 mm short of the next region. The typhlosole is formed from an internal dorsal fold in the epithelium which has lateral expansions curving dorsally to form two parallel channels along the dorsal surface of the gut (Figs. 4 and 5). The epithelium forming the typhlosole is not differentiated into cell types and, like the epithelium lining the remainder of the anterior hindgut, consists of columnar cells 60–35 μm wide, with single dense nuclei, lightly basophilic cytoplasm and with the chitinous intima closely molded to their distal surfaces which protrude somewhat into the gut lumen (Fig. 6). They show no reaction for iron, esterases or arylamidases. All the cells, though, apart from those lining the channels of the typhlosole, show a thin distal zone of both acid and alkaline phosphatase activities (Fig. 5).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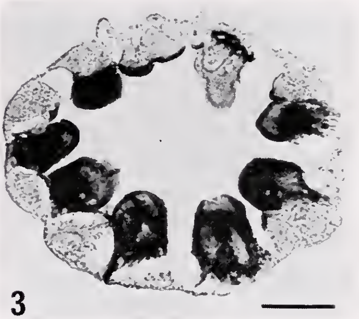
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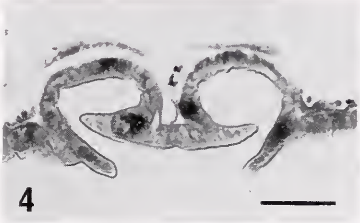
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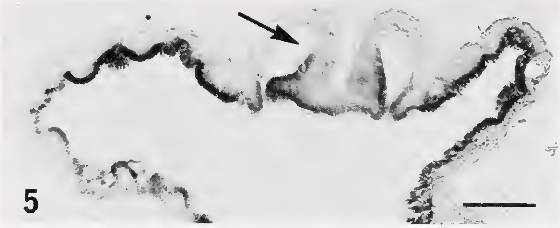
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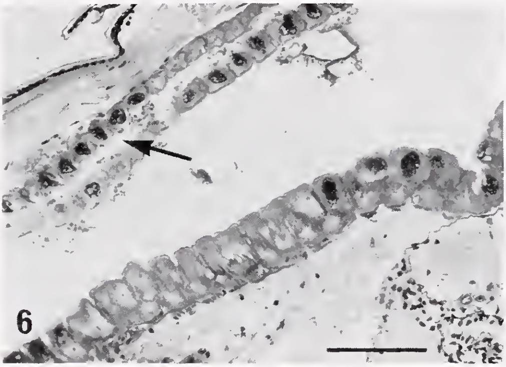
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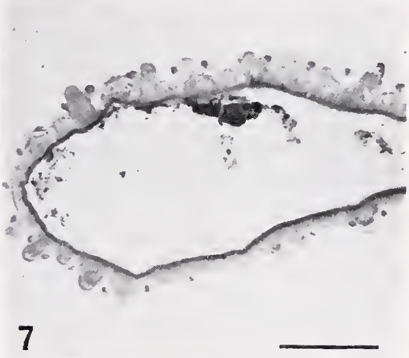
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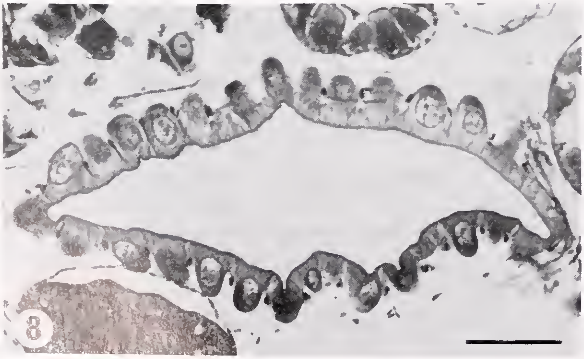
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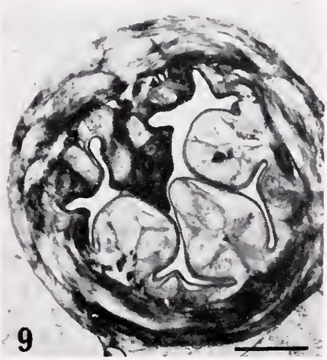
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The muscle layers of the gut wall do not enter the typhlosole. Both layers are equally developed and form a thin uniform envelope around the epithelial layer.

The externally papillate region. The second portion of the hindgut is 1.5–1.8 mm in length and externally has a characteristic papillate appearance (Fig. 1). The "papillae" are, in fact, the constituent cells of the epithelium whose basal regions project outwards into the haemocoel (Figs. 7 and 8). The cells are all of the same type, 60–70 μm tall and 35–40 μm broad, each with a large prominent nucleus and basophilic cytoplasm. The basal regions projecting into the haemocoel contain large amounts of organically bound iron, comparable to those occurring in the caecal cells, but no parts of the cells show any reactions for esterases or arylamidase. There is, however, a distal zone of acid and alkaline phosphatase activity, 2–4 μm deep, and a general but weaker reaction for acid phosphatase is given by the rest of the cytoplasm (Fig. 7).

The circular muscles show no modification in this region of the hindgut, but the longitudinal muscles become separated into distinct blocks which lie between the protruding bases of the cells (Figs. 7 and 8).

The sphincter region. The papillate region is separated from the rectum by a portion of hindgut 0.6–0.7 mm long in which the band of circular muscles in the gut wall increases in thickness from the 2–3 μm characteristic of the rest of the hindgut to 90–100 μm (Fig. 9). The longitudinal muscles remain unchanged but the epithelial layer is thrown up into pear-shaped folds which interlock when the circular muscles contract and thus seal off the papillate region from the rectum.

The muscular layers show a uniform reaction for acid phosphatase but the epithelial layer shows no enzymic reactions and does not contain iron.

The rectum. The terminal portion of the hindgut is 1–1.2 mm long and opens to the exterior at the slit-like anus. The muscular layers are unmodified; the epithelium is folded but not to the same extent as in the sphincter region and the

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FIGURE 2. *P. muscorum;* longitudinal section of a caecum showing differential distribution of iron (black) in the epithelial cells; ammonium sulfide, potassium ferrocyanide and hydrochloric acid technique; scale = 100 μm.

FIGURE 3. Transverse section of a caecum showing esterase activity (black) in the epithelial cells. Section is treated by Holt's method for non-specific esterase; scale = 50 μm.

FIGURE 4. Transverse section through the median dorsal region of the anterior hindgut showing the typhlosole. Feulgen and fast green; scale = 200 μm.

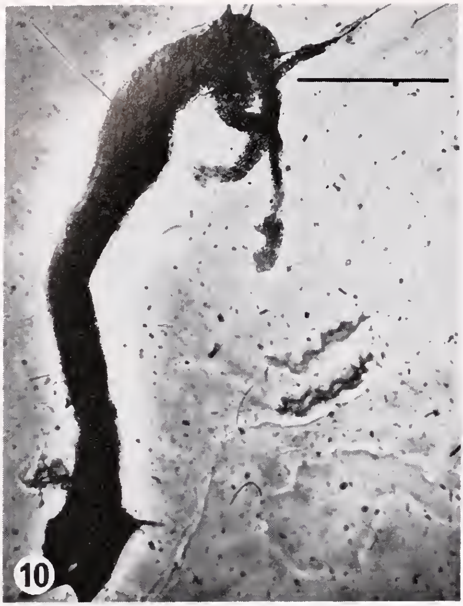
FIGURE 5. Transverse section through the anterior hindgut showing alkaline phosphatase activity (black) in the distal regions of all epithelial cells except those lining the typhlosole channels (arrowed). Section is treated by Burstone's method for alkaline phosphatase; scale = 200 μm.

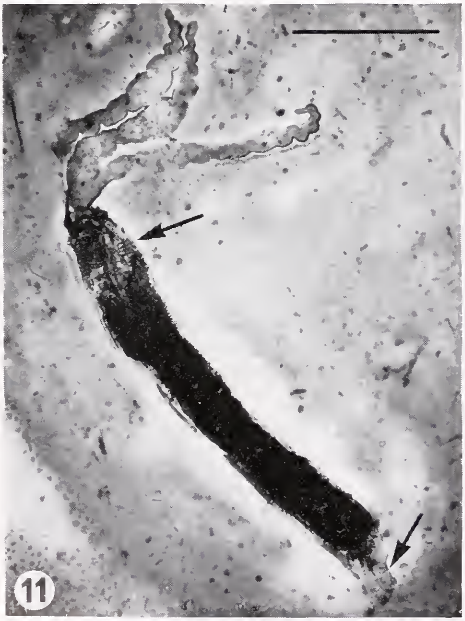
FIGURE 6. Longitudinal section through the anterior hindgut showing the ventral epithelium (cut obliquely) and one of the channels (arrowed) formed by the typhlosole. Section is stained with haematoxylin and eosin; scale = 200 μm.

FIGURE 7. Transverse section of the papillate region of the hindgut showing a diffuse reaction for acid phosphatase throughout the cytoplasm and a stronger distal band. Section is treated by Burstone's method for acid phosphatase; scale = 250 μm.

FIGURE 8. Transverse section of the papillate region showing the epithelial cells protruding outwards into the haemocoel, with longitudinal muscle blocks lying between them. Section is stained with Mallory's triple stain; scale = 150 μm.

FIGURE 9. Transverse section of the sphincter region of the hindgut showing the increase in thickness of the circular muscles and the pear-shaped folds of the epithelium. Section is stained with Mallory's triple stain; scale = 100 μm.





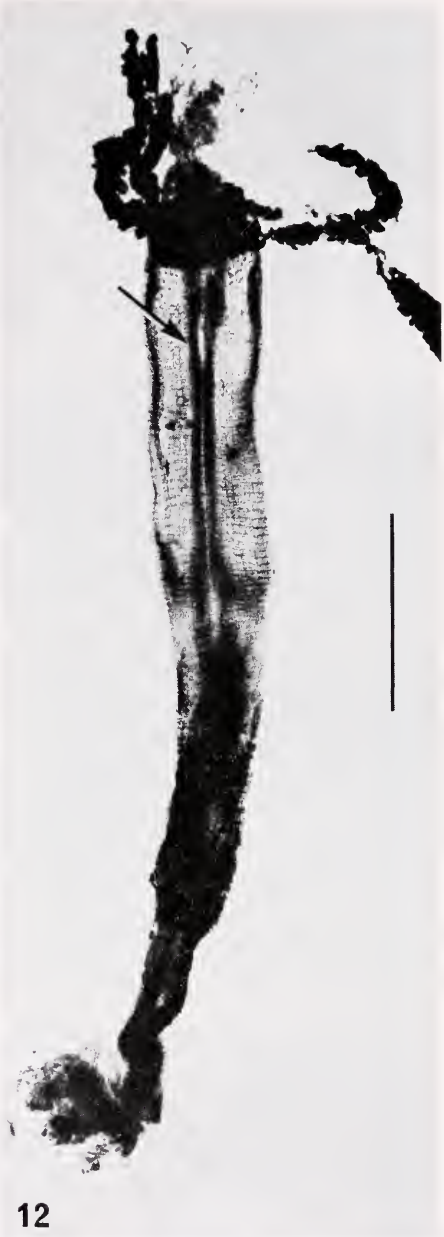


FIGURE 10. *P. muscorum;* alimentary canal fixed after a meal of decomposing leaf litter, covered with a thin film of carboxymethyl cellulose, incubated for one hour and subsequently stained in toluidine blue. Parts of the film hydrolyzed by cellulases diffusing from the specimen appear white, while unchanged areas appear grey; scale = 2 mm.

FIGURE 11. As Figure 10 but showing reduced cellulase activity in those regions of the hindgut (anterior hindgut and rectum, arrowed) which contain little or no food. Areas of the

chitinous intima is thicker and stains more heavily with periodic acid-Schiff. The epithelial cells contain both acid and alkaline phosphatases uniformly distributed throughout their cytoplasm, but give no reaction for esterases, arylamidases or iron.

Cellulases

Intact alimentary canals from starved animals and from individuals fed for at least 5 days on boiled potato showed no reaction when tested by Sumner's substrate film method, indicating that there are neither endogenous cellulases nor a permanent symbiotic microflora producing cellulases in *P. muscorum.* In marked contrast, though, very strong cellulase activity was found in the fore- and hindguts of specimens which had ingested the natural food of decaying leaf litter, but the caeca consistently gave negative results (Figs. 10 and 11). Confirmation that the cellulases responsible for digestion of the substrate film were already present in the food, before ingestion, came from testing uningested food, which showed a positive reaction, and from examination of alimentary canals from isopods which had taken only small meals. Such specimens had regions of the gut which contained little or no food (*e.g.* the anterior portion of the typhlosole region of the hindgut and the rectum in the specimen shown in Fig. 11) and cellulase activity was either reduced or absent in these areas.

The incubation time in all cases was standardized at one hour to allow some comparison of the relative degrees of activity in the food before and after ingestion. So far as could be judged the food showed greater cellulase activity after being confined in the hindgut for 6–12 hours, indicating that there is perhaps some factor operating there which favors growth or enzyme production by the microorganisms. Alternatively the increased activity may have been due simply to closer packing of the food materials, resulting in increased numbers of microorganisms per unit volume as compared with the loose uncompressed state of the food before ingestion. Freshly passed faeces, however, also showed higher levels of cellulase activity but these decreased with time until 6–7 days later when they were the same as those seen in samples of the natural food.

Heat inactivated preparations of the food, and of alimentary canals from fed individuals, showed no cellulase activity.

A single starved isopod was induced to ingest a freshly prepared liquid paste of the carboxymethyl cellulose used in the substrate film investigations. It was fixed 12 hours after feeding in the acid ethanol used during preparation of the substrate films and subsequently stained with toluidine blue. Small amounts of unchanged carboxymethyl cellulose were seen in the lumen of the caeca, providing confirmatory evidence for the absence of endogenous cellulases from the caeca and for the entry on at least some occasions of fluid materials into these structures.

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film adjacent to the caeca show no hydrolysis; the narrow lighter zone adjacent to the lower caecum is the result of refraction of light by the cellulose film during photography and in the original preparation appeared as blue as the remainder of the unchanged film; scale = 2 mm.

FIGURE 12. Alimentary canal fixed 24 hours after a meal of boiled potato, showing intense esterase activity (black) in the caeca, proventriculus, typhlosole channels (arrowed) and papillate region. Section is treated by Holt's method for nonspecific esterase; scale = 2 mm.

Digestion and absorption

The course of digestion was followed in sections of alimentary canals from isopods fixed at progressive intervals up to 7 days after they had fed on boiled potato devoid of intrinsic enzymes. During feeding the proventriculus rapidly filled with potato and then gradually emptied over the next 5–10 minutes as the contents were passed onwards into the hindgut. The proventriculus then filled again and the process continued until the anterior hindgut and the papillate region were packed. At this point the isopods stopped feeding and the proventriculus remained empty.

The food developed a slight but distinct esterase reaction as it passed into the the hindgut. The reaction showed the same responses to activators and inhibitors as those given by the esterases seen in the caecal cells and it is concluded therefore, that the caeca discharge a small amount of enzymes, exemplified by esterases, onto the food as it passes through the proventriculus. Support for this came from the observation that the esterase reaction in the caecal cells declined in intensity during feeding, but then gradually returned to normal over the next six hours.

The food lying in the hindgut continued to show a low level of esterase activity until 20–24 hours after ingestion. About this time the contents of the papillate region showed a marked increase in esterase activity and this coincided with the appearance of esterases in the two channels formed by the lateral expansions of the typhlosole (Fig. 12). Esterases also appeared in the proventriculus so that there was a continuous band of enzymatic activity between the caeca and papillate region. Sections of the typhlosole showed that the enzymes were not originating in the endothelial layer and it is concluded that they are secreted by the caecal cells, poured into the proventriculus and directed by this into the two channels of the typhlosole. They thus bypass the anterior hindgut and are discharged from the posterior end of the typhlosole onto the food lying in the papillate region.

During the 3–4 days after this increase in esterase activity material moved slowly from the papillate region into the rectum and was then voided as faeces. It was replaced by material from the anterior portion of the hindgut, which in turn developed increased esterase activity as it entered the papillate region, and this process continued until the hindgut was completely empty some 7 days after the observed meal. It is presumed that under natural conditions, though, the gut would rarely be empty and that there would be a slow but steady movement of material through the alimentary canal, with regular additions to it of esterases, *via* the typhlosole, in the papillate region.

The caecal cells normally show some reactions for acid and alkaline phosphatases and arylamidases but these enzymes were never demonstrated in the secretion passing along the typhlosole canals. Some slight arylamidase activity did develop, however, in food as it passed through the proventriculus and this persisted for as long as the material remained in the hindgut. The enzyme, or enzymes, responsible are presumably secreted by the caecal cells along with the esterases added to the food, soon after ingestion, in the proventriculus.

Treatment of sections of potato-fed isopods with Lugol's iodine or periodic acid-Schiff showed that there is no hydrolysis of starch in the proventriculus. A small amount of hydrolysis was seen in the typhlosole-bearing portion of the hindgut but the largest amount was found in the papillate region. At no time, though, was there hydrolysis of the entire meal and in all specimens examined there were always considerable quantities of unchanged starch in the hindgut. The faeces, similarly, always contained much starch and it would appear that *P. muscorum* has only a low assimilation efficiency for this type of food, and, indeed, for the natural food, much of which passes through the alimentary canal apparently little changed.

The high level of esterase activity in the papillate region suggested that this is the principal site of endogenous enzyme activity and since phosphatases occur in the distal portions of the epithelial cells it was thought likely that this part of the hindgut may be the principal site of absorption of digestive products. Phosphatases occur in the epithelium of the typhlosole region but not in that lining the typhlosole channels (Fig. 5) and in the caeca, so that absorption would be expected at these sites also. Attempts were made to demonstrate absorption by using saccharated ferrous carbonate as a component of test meals, but the results had to be interpreted with caution. Control sections of both starved and potato-fed individuals showed that the epithelial cells of the caeca and papillate region normally contain substantial quantities of iron, as described earlier, so that absorption of iron or iron-containing compounds by these cells in the specimens fed saccharated ferrous carbonate could only be deduced if they showed a significant increase above this pre-existing level. Further, the controls in which the saccharated ferrous carbonate was injected mechanically into alimentary canals that were then fixed immediately and processed, showed that diffusion artifacts could arise where large amounts of the carbonate were present in the gut lumen. Despite these limitations, there was clear evidence of absorption in the caeca and in the anterior and papillate regions of the hindgut, with the epithelial cells showing slight but significant increases in iron content. The most significant result derived from these particular feeding experiments, though, was the appearance of small amounts of saccharated ferrous carbonate within the lumen of the caeca without any traces of the potato tissue which had formed the bulk of the test meal and which was present in the proventriculus and hindgut. This gave further confirmation that components of a meal can be separated by the proventriculus and that substances in solution can be diverted into the caeca.

DISCUSSION

Three salient points emerge from this study of gut structure and function in the terrestrial isopod *Philoscia muscorum*. These are that the caeca are confirmed as the major source of endogenous digestive enzymes; that the function of the typhlosole is to conduct enzymes from the caeca to the papillate region of the hindgut and thereby allow them to bypass the anterior hindgut; and that the cellulases operating in the gut are already present in the food before ingestion and are not produced by the animal or by permanent symbiotic gut flora.

The separation of liquid components from the food by the proventriculus and their diversion into the caeca, described by Hassall (1975b) and confirmed here, presumably occurs during or very soon after ingestion since there is also flow of enzymes in the reverse direction, from the caeca, onto the solid food as this passes into the hindgut. It seems unlikely, though, that there will be much digestion in the caecal lumen as solids have never been found there. The liquids derived from the natural food probably contain simple substances which require little or no treatment before absorption, and the capacity for absorption in the caeca was fairly clearly demonstrated in the saccharated ferrous carbonate feeding experiments. It may well be that the caeca are major sites of water absorption, and perhaps water storage, and that absorption of nutrients in this part of the gut is of only secondary importance.

There is no obvious differentiation of the caecal epithelium into secretory and absorptive cells; the differences in the size and shape of the constituent cells have been interpreted, in other species, as indicating the presence of at least two types of cell (Murlin, 1902; Chandy, 1938; Alikhan, 1969; Clifford and Witkus, 1971; Hryniewiecka-Szyfter, 1972) but an alternative view is that only one type is present and that the differences simply reflect different stages in its growth (Beecher-Moore, 1956; Donadey, 1969; Jones, Babbage and King, 1969; Schmitz and Schultz, 1969). The range of cell sizes, and the direct relationship between size on the one hand and basophilia and iron and esterase content on the other, seen in *P. muscorum* suggest that for this species, at least, the second interpretation is correct. It seems likely that the smaller cells are, in fact, younger cells which go through an initial absorptive phase, in which they take up material from the caecal lumen, before they mature and assume a predominantly secretory function. During this second phase the cells produce digestive enzymes, exemplified by esterases, which affect hydrolysis of food principally at sites other than the caecal lumen.

The origin and physiological role of the iron deposits in the caecal cells and, indeed, in those of the papillate region, are unknown; similar accumulations of this metal and of copper are a characteristic but as yet unexplained feature of isopod gut caeca (various authors, summarized in Wieser and Klima, 1969; Alikhan, 1972; Hryniewiecka-Szyfter, 1972).

The caecal enzymes operate at two separate points in the digestive process. They are secreted first in only small amounts onto the food as it passes through the proventriculus and remain active, albeit at a relatively low level, for the next 20–24 hours. The consistently low level indicates, incidentally, that there is no supplementary secretion of esterases from the hindgut endothelium. The second and major addition of caecal enzymes to the food occurs at the end of this period but, unlike the first and minor secretion, the enzymes are not discharged onto food in the proventriculus or anterior hindgut. Instead, they are diverted *via* the proventriculus into the typhlosole channels, bypass the anterior hindgut and are discharged onto material lying in the second, papillate, region of the hindgut.

The functional and adaptive significance of the typhlosole and of the subdivision of the hindgut into anterior and papillate regions, now becomes apparent. *P. muscorum*, in common with other terrestrial isopods possessing these features, feeds on decomposing plant materials rich in microorganisms which produce, among other enzymes, cellulases. The modification of the alimentary canal allows the isopods to exploit to the fullest the degradative capabilities of these free-living microorganisms in their own utilization of leaf litter as food. Any readily available nutrients in solution at the time of ingestion are passed into the caeca and absorbed. The bulk of the meal, though, passes into the anterior hindgut and possibly also into the papillate region where the microbial enzymes, including the cellulases demonstrated in the present study and together with a small quantity of the isopod's own caecal enzymes, continue the breakdown of the food initiated before ingestion. Under laboratory conditions with previously starved animals this occupied up to 24 hours; under natural conditions this period may vary and be dependent on the quantity and quality of the food available. Whatever the duration, though, the typhlosole allows the isopod eventually to discharge more endogenous enzymes onto the food after a post-ingestive continuation of microbial attack.

The anterior chamber of the hindgut occupies about one half of the total length of the hindgut; it contains the bulk of ingested food and so is the major site of action by exogenous enzymes. It is significant, then, that the typhlosole delivers the endogenous enzymes at a point posterior to this site. This enables them to carry out their particular digestive functions on food already subjected to extended microbial attack, without them interfering with any further microbial action in the anterior hindgut, either on material remaining there after some has moved on into the papillate region or on newly ingested food.

The possibility that the typhlosole is concerned in some way with movement of digestive enzymes was originally put forward by Murlin (1902). He was unable to test it, with the techniques then available, and it was virtually ignored by subsequent investigators.

The distribution of phosphatases in the anterior and papillate regions of the hindgut, and the results of feeding saccharated ferrous carbonate, indicate that absorption occurs in these areas. This is to be expected, of course, since these areas are known to be sites of digestion. Phosphatases are absent from the typhlosole channels, which is understandable if these structures are used only to transport caecal enzymes. If the iron which is always present in some parts of the alimentary canal is the result of the accumulation over a long period of small amounts absorbed from the diet, which is feasible, then its differential distribution may well be instructive. Its occurrence in the caeca and papillate region could indicate that *any* soluble substances derived from the food can be, and are, absorbed in these areas. Its absence from the anterior part of the hindgut, in contrast, suggests that only products of cellulose digestion are absorbed here, making this region directly comparable to the anterior chamber of the stomach in ruminant mammals.

Previous attempts to detect cellulases in isopods, and determine their origin, involved application of biochemical, viscometric or microbiological techniques to gut extracts and yielded conflicting results. Nicholls (1931), with *Ligia oceanica,* and Newcomer (1956) with *Porcellio* sp. were unable to detect any cellulase activity, while Ray and Julian (1952) and Ray (1959a; 1959b) with *Limnoria lignorum* concluded that the cellulases they found in the caeca and elsewhere in the gut were secreted by the isopod since they found no evidence of microorganisms anywhere in the gut. Hartenstein (1964) demonstrated cellulases in caecal and whole gut extracts from *Oniscus asellus* and likewise believed them to be endogenous, although his specimens were fed on decaying sugar maple leaves. In this last study the 'caecal cellulase' could have been present in liquids pressed out of the food and passed into the caeca, although admittedly this was never found in the present work.

The cellulose-splitting activities of the microorganisms ingested by *P. muscorum* seem to be somewhat enhanced in the isopod's gut, as compared with those in control samples of uningested food. The reason for this is not immediately apparent, but it is interesting to note that similar enhancement of microbial activity has been observed in larval Bibionidae (Diptera) where two or three of the bacterial species ingested with the food proliferate in the favorable gut environment and assist digestion (Szabó, Marton and Buti, 1969). Selective survival of ingested microorganisms, and utilization of their hydrolytic capacities, occurs also in predatory leeches (Jennings and van der Lande, 1967) although here there is no particular enhancement of microbial activities.

The persistence of high cellulase activity in the freshly passed faeces of *P. muscorum* suggests that the endogenous enzymes acting in the papillate region do not kill the microorganisms. This is an interesting difference from the situation in those herbivores such as ruminant mammals which, while similarly utilizing microorganisms for cellulose digestion, and facilitating the process by delaying onset of endogenous digestive activity, do eventually digest the microorganisms and use them as a source of protein. In these instances the microorganisms are mainly symbiotic species and not, as in *P. muscorum*, free-living species ingested with the food. In most animals, of course, such micoorganisms are normally killed after ingestion. Alternatively it may be that only the microbial cellulases resist digestion in the isopod hindgut and that the microorganisms are killed and utilized as food. This would be a more efficient process and the possibility will be examined in future work.

Those features of the isopod alimentary canal which are peculiar to terrestrial species, namely the typhlosole and the subdvided hindgut, are thus believed to have a very definite functional and adaptive significance. The significance of the reduction of the midgut to the caeca, though, which is characteristic of all isopods, remains obscure. Midgut caeca are common in invertebrates, and where they play major parts in digestion and absorption the remainder of the midgut, and often the hindgut too, are reduced in size and importance (Jennings, 1972). In isopods, though, that part of the alimentary system posterior to the caeca remains fully functional and, in terrestrial species at least, has increased in importance and become the region in which the bulk of the digestive process occurs.

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SUMMARY

1. The alimentary canal in the terrestrial isopod *Philoscia muscorum* consists of a foregut (oesophagus and proventriculus), a midgut reduced to four simple caeca, and a hindgut subdivided into anterior, papillate and sphincter regions and a rectum. The anterior region has a dorsal typhlosole whose lateral extensions form two parallel channels beneath the roof of the hindgut.

2. The isopod feeds on decaying leaf-litter. Liquids are separated in the proventriculus and passed into the caeca and absorbed; solids receive a small amount of digestive secretions containing A- and C-esterases, from the caeca, as they pass into the anterior hindgut.

3. Microorganisms already present in the food and responsible for its degradation prior to ingestion continue their action in the hindgut. They produce cellulases demonstrable by a substrate-film technique and it is believed that they are the only source of the cellulases present in the alimentary system. There is some indication that microbial activity is enhanced after ingestion by the isopod.

4. Microbial attack on the food in the anterior hindgut lasts for 20–24 hours under laboratory conditions and is followed by the major discharge of caecal enzymes. These now bypass any food remaining in the anterior hindgut, or recently ingested and passed into it, by being carried along the dorsal typhlosole channels. They are discharged into the papillate region and attack food which has already been subjected to extended microbial action.

5. Absorption occurs in both the anterior and papillate regions of the hindgut. It is suggested that only products of cellulose digestion are absorbed in the former while other digestive products are absorbed in the papillate region.

6. The typhlosole and subdivision of the hindgut are interpreted, therefore, as adaptive modifications related to a diet of decomposing leaf-litter and the utilization of the digestive capabilities of normally free-living microorganisms for the breakdown of this food, and especially of its cellulose component.

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NONHETEROSEXUAL BEHAVIOR IN MOSQUITOES[1]

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Males of *Aedes aegypti* mosquitoes specifically use their antennae as phonoreceptors to pick up the buzzing sounds of the vibrating wings of flying females (Roth, 1948). Sound is the primary sexual cue given to the male. He must critically perceive sound to recognize the very presence of a female and then, still using sound, normally may locate and then choose a female with great precision and speed as she flies through the air (Jones, 1974). Males rapidly chase and capture females on the wing. Recognition, location and capturing of the female all depend on sound. Using his tarsi, the male quickly orients himself ventrally to the female and seizes her terminalium with his claspers. After terminalial contact, the aedeagus of the male may be inserted into the vagina and after this he may ejaculate into her bursa (Jones and Wheeler, 1965).

In his detailed study on the sexual activities of *Aedes aegypti*, Roth (1948) observed what he referred to as being "homosexual" behavior among the males, and he reported very high rates in the 2 counts he made on his strain. Male couples can be seen in colony cages in the laboratory under routine conditions.

It was the purpose of the present study to reexamine the sexual behavior of male mosquitoes relative to one another under various experimental conditions.

MATERIALS AND METHODS

The Bangkok and U. S. Naval Medical strains of *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) were mostly studied under free-flying conditions in a one cu ft cage with clear plastic sides in an insectary at 27° C and 70% relative humidity. All observations were made between 0830 and 1030 hours. The term "sexual encounter" includes a great variety of different acts: a distinct chase with or without capture, a midair capture without genital contact, a midair capture with genital contact, and capture with subsequent landing (with or without genital contact). Since it was not possible to observe individual couples in a large group for more than 10 to 15 sec, the same couple could have been recounted at least twice, especially if a couple were flying or if they landed and reflew as a couple. Ten percent sucrose was available except during the periods of observation and experiment.

The following terms and their definitions are critical. *Homosexuality* is well-defined as attraction towards members of the same sex (Dorland, 1974). The key word in this definition is attraction: the homosexual animal must perceive the other animal as being of the same sex and proceed towards it. For true homosexuality to occur, a specific choice of one sex must be made and the opposite sex avoided (White, 1963). *Heterosexuality* is defined here as attraction towards individuals of the opposite sex, with no attraction towards individuals of the same sex. *Bisexuality* is defined as equal attraction towards individuals of both sexes. *Nonheterosexual* behavior refers to mating activities between members of the same sex which are neither homosexual nor bisexual in character. *Copulation* will be defined as the insertion of a copulatory organ into an animal orifice.

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RESULTS

General flight activities of males

One day after emergence, adult males of *A. aegypti* either rest on the sides of a one cu ft cage or fly for relatively long periods of time. They normally fly quite rapidly in the confines of a one cu ft cage and make zigzagging flights as they move from side to side or fly up or down. They readily make sudden looping flights and can swiftly fly downwards for 10–20 cm without landing. Males fly very fast when they give chase to other mosquitoes. They appear to slow up as they first turn to follow another individual. Males in flight manuever with great speed and agility, and, in the majority of cases, make no attempts to capture each other, when they are of the same age. Males can fly very briefly "face to face." In a cage containing 100 males they often come within about 3 mm of each other and then dart away.

Description of nonheterosexual behavior

Nonheterosexual behavior was exhibited only among adult males and was never observed between females.

In a cage containing only males, single individuals can be seen suddenly chasing another male. Such chases often do not result in capture by the agressive male. Midair captures of one male by another were often seen where the couple very quickly broke off all contact just before they landed on the floor of the cage. Only very rarely would a male couple continue to fly together for longer than 5 sec. Many male couples were observed to fall to the floor of the cage and to be grossly misaligned relative to each other. Such couples usually parted after a brief struggle or after a variable period of mutual catatonia. Sometimes, however, one of the aggressive males would quickly thrust his terminalium in the general direction of the other, without making any terminalial contact with the passive partner. Aggressive males were generally located ventral to the passive male or else along one side. When a male couple happened to land so that the aggressive partner was on top of a captured male (which landed on his back), the top-mounted male made no terminalial thrusts, and generally quickly flew off. In the most clear-cut form of nonheterosexual behavior, the aggressive male first chases and quickly captures a passive male and the couple land on the floor with the aggressive partner ventral to and aligned with the passive male. Then, the aggressive male quickly seizes the claspers of the other male in such a way that very close genital contact occurs. Genital contact among such free males is always very brief and lasts about one sec or less. The aggressive male may make repeated efforts to make genital contact and the couple may struggle on the floor for a relatively long time, turning over and over. The couple will sometimes fly for a short distance with the aggressor still attempting to grasp the other male's terminalium. In between these struggles, the couple may stand in mutual catatonia. Eventually, the struggles lead to separation of the pair.

It was frequently observed that the aggressive males usually did not have distended abdomens and that they would very commonly capture those older males which had their abdomens distended with sugar solution.

Sometimes in a cage of mixed sexes, a female would attract 2 or sometimes 3 males and after these had fallen to the floor, one of the males would usually copulate with the female, and, afterwards, this aggressive male might make a series of abdominal thrusts at the male who happened to be closest to him in the group. Sometimes 2 out of 3 males in association with one female would make abdominal thrusts at each other.

Levels of sexual activity in an all-male population

One hundred newly-emerged males were placed in a one cu ft cage and were observed over a period of nearly 12 days. Observations were usually made on undisturbed individuals for a 30 min period on each day they were studied. On the first six days, the animals were also deliberately disturbed, generally at one min intervals over another 30 min period. As shown in Table I, no nonheterosexual behavior was observed among 100 males during the first 24 to 36 hr under undisturbed cage conditions. After this time (from 48 to 276 hr), 10 to 20 nonheterosexual encounters were observed in a 30 min period. Deliberate shaking of the cage to disturb the males increased the number of males flying only for a brief period of time and did not specifically increase the level of nonheterosexuality in the cage.

TABLE I

Nonheterosexual encounters in an all-male population of Aedes aegypti.

Age of males	Number in cage	Male couples	
		Undisturbed cage 30 min	Disturbed cage 30 min
0–12 hr	100	0	0
3–15 hr	100	0	0
24–36 hr	100	0	2
48–60 hr	99	10	12
72–84 hr	99	20	11
96–108 hr	99	14	11
168–180 hr	99	17	—
192–204 hr	99	15	—
216–228 hr	96	20	—
240–252 hr	95	17	—
10 newly-emerged males added to cage		—	16
24–276 hr	104	19	—

Effect of isolating male couples on sexual activity

When the all-male population in the cage was 3 days old, those couples which were found together on the floor of the cage were quickly aspirated into another one cu ft cage. In all, 20 couples were so isolated. All 40 males flew very actively back and forth and often came very close together yet none of them made any attempts to chase or capture other males during an hour of careful study. This finding specifically and clearly demonstrates that the activity seen in the larger cage is not true homosexuality. When 40 virgin females were placed in the cage, there was a very great increase in sexual activity, and the females were quickly captured by the males and copulated with. Thirty min later, when the females were dissected, all of them had been inseminated and impregnated.

Experimentally increasing the nonheterosexual rate in a male population

The maximum level of nonheterosexual activity at any one moment in the all-male population under either undisturbed or disturbed conditions varied from 3 to 4 couples. When a tuning fork of 512 cycles per sec was struck within the cage, the males formed dense swirling masses around the fork, and there was a very marked increase in nonheterosexual captures during the short time the fork was actually vibrating. As many as 11 male couples were counted within 32 sec after striking the tuning fork. As many as 9 couples were seen on the floor of the cage at the same time. This was the maximum number of couples seen at any one time in this study. These male couples were nearly all very loosely attached only by one tarsus, and the insects stood side by side. Most couples made no attempts to establish genital contact and quickly broke up. Sometimes a group of 3 males was seen together.

When the males were 10 days old, the undisturbed nonheterosexual rate was 17 couples within a 30 min period. After this, 10 newly-emerged males were added to the cage and the number of male couples were counted over the next 30 min period. The nonheterosexual rate for this period was 16; that is, there was no increase in the rate. It was observed that many of the young males were indeed preferred to the old ones. It was also noted, however, that the older males frequently did not attempt to establish genital contact with them, even though they had been captured.

Since newly-emerged adult males are not only reluctant to fly at all but fly only for very short distances, it was of great interest to learn how it was possible for them to be captured at all by older males. It was soon observed that older aggressive males often landed very close to the newly-emerged males shortly after they had been introduced into the cage. The older males generally had one tarsus in contact with one of the legs of the young males. Thus, as soon as the young male flew, he would be immediately accessible to capture. Aggressive males also frequently land beside females in this way and more quickly capture them as well.

The young males were left in the cage overnight, and the next day a nonheterosexual rate was taken and estimated to be 19 couples in a 30 min period.

Ten 3-day old virgin females were then placed in the cage with 104 males. When heterosexual and nonheterosexual rates were counted over a 30 min period.

it was found that there were 71 heterosexual contacts and 142 nonheterosexual encounters within the first 30 min of placing the females in the cage. There was, thus, an enormous increase in general sexual activity. Although the nonheterosexual rate increased 8.6-fold, the maximum number of male couples on the floor at any one time was only 5. A number of male trios were also seen, far more than under any other experimental conditions. Some individual females were also picked up by 3 to 4 males at one time.

An attempt was made to compare the relative "attractiveness" of 5 recently-emerged young males with 5 3-day old females to a group of 10 3-day old males. A good comparison was not possible because most of the young males did not fly, whereas most of the females were very active fliers. First, 5 females were placed in a one cu ft cage and then 12-hr old males were introduced. None of the young males chased the flying females for a 30 min period. Only 2 of the 5 young males flew briefly when the cage was disturbed. A group of 10 3-day old males was then placed in the cage. Within 30 sec one male couple was seen with the aggressive male making repeated terminalial thrusts for a relatively long period of time. During the 30 min period of observation, this was the only male couple seen. During this same period, there were 63 heterosexual encounters.

Sexual activity in a heterosexual population

The final proof of a state of nonheterosexuality versus homosexuality would be to show that when there was an equal number of sexes of the same ages within a given population, there would be a very marked decrease in male couplings. As shown in Table II, when 50 males were placed with an equal number of females when they were less than one day old, heterosexual behavior was the predominant or only sexual activity seen over an 11 day period. Since the level of sexual

TABLE II

Extent of heterosexual and nonheterosexual activity in a population of Aedes aegypti *when the sex ratio is 1:1.*

Age of the mosquitoes	Number in cage	Number of encounters in 30 min	
		Heterosexual couples	Possible nonheterosexual couples*
0–18 hr	100	7	1
24–48 hr	100	74	1
48–66 hr	100	85	5
72–100 hr	99	85	3
96–124 hr	99	99	6
168–196 hr	98	81	1
192–220 hr	96	294	5
216–244 hr	90	224	1
240–268 hr	89	203	1

* Since the males were very similar in size to the females in this population, and since male couplings were very brief, it was not possible to make accurate counts. Most of the values given in this column were noted as being possible male couplings.

activity was very high under undisturbed conditions, no deliberate attempts were made to disturb the mosquitoes. None of the male couples showed any attempts to seize the other's terminalium.

Discussion

The word homosexuality is often used very loosely to refer to a great variety of undefined associations and ill-defined acts involving individuals of various species of the same sex. If the word homosexuality is defined as being a specific attraction towards members of the same sex (Dorland 1974), with avoidance of the opposite sex (White, 1963), then it is evident that male mosquitoes do not exhibit homosexuality. That is, when these animals are given a specific choice, they choose females and specifically avoid other males under heterosexual conditions. Even when the males are kept continuously in an all-male group, most of them specifically avoid each other. The level of male couplings in the all-male population used in this study was estimated as involving about 16% of the individuals over a 30 min period under undisturbed conditions in a one cu ft cage. One-half of these animals were aggressive males (that is, they captured other males which were their passive partners). This means that only about 8% of the population actually chase and capture other males. Since one aggressive male may have repeatedly captured one or more males, he could easily have been counted on more than one occasion during a 30 min period, so the 8% level of aggressive males is probably too high, by a factor of at least 2.

The estimated level of nonheterosexual behavior in an all-male population in the presence of newly-emerged males in a 30 min period under disturbed conditions is extraordinarily less than that recorded by Roth (1948) where he counted 98 male couplings within a 15 min period among only 22 males (4 young males with 18 old males). This averages about one sexual encounter every 9.2 sec! If each of the 4 young males were captured uniformly by older males (which is most unlikely), this would mean that each young male would be picked up about 25 times in a 15 min period.

In the present work, only 16 male couples were seen within a 30 min period among a group of 105 mosquitoes, where there were 9.5 times as many old males as young ones.

Roth (1948) believed that *A. aegypti* males would sometimes attempt to "copulate" with each other. Jones and Wheeler (1965) rubbed the terminalia of 2 restrained males together and observed that although one male would sometimes grasp the claspers of another with his own claspers, the aggressive male never erected his paraprocts or aedeagus, thus indicating that actual copulation between 2 males does not in fact occur. Observations made during the present studies support the observations of Jones and Wheeler (1965).

It is of considerable interest that the majority of aggressive males in nonheterosexual encounters seem to recognize they do not have a female partner before terminalial contact is made and quickly separate before landing occurs. Some more aggressive males capure another male and make terminalial thrusts and then depart without any terminalial contact beween the partners. Some aggressive males seize the terminalia of their partners a single time and at that moment seem to recognize they do not have a female. A very few aggressive males make repeated terminalial thrusts and may repeatedly seize the claspers of another male before they recognize they are not in contact with a female.

In many animals the males do not recognize the sex of their partners until after the capture (Beach, 1938). The biological "philosophy" of a trial-and-error method is probably: It is better to grab and take a chance on having the right partner than no partner at all. Only the most aggressive males of *Aedes aegypti* seem to have this "philosophy"; most males avoid other males and most of them choose females with considerable accuracy. They do this by sound alone. The remarkable thing is that with this one sexual clue, the males make so few mistakes. Apparently, the males receive some additional cue from females prior to copulation, and this cue is missing from males.

It is concluded that a relatively low level of nonheterosexual activity occurs in most populations of *Aedes aegypti*. This phenomenon is the result of a momentary misperception of sound by aggressive males which mistake other males for females.

The most striking feature of nonheterosexual activity in *A. aegypti* is that most of the aggressive males which have captured another male make no attempts to orient themselves to the body of passive partner. This failure seems to be due primarily to the fact that most aggressor males do not land in a ventral position relative to their partners. If the aggressive males, however, land in a ventral position relative to their partner, then they often will make attempts at alignment and may thrust their abdomens in the direction of the other. In most cases, however, ventrally oriented aggressive males do not make genital contact. In a few cases, however, the aggressive partner not only attains a ventral position but is correctly aligned and actually establishes genital contact, and may do this more than once with the same male. In all such cases, however, the terminalial contacts were very brief (for less than one sec), strikingly more so than genital contacts made with free-flying females, where the unions vary from 1.2 to 84.6 sec (averages of 11 sec for 55 cases) (Jones, 1974). Males may be misaligned relative to captured females but they very often correct the misalignment and copulate.

Nonheterosexual activity differs in many ways from heterosexual behavior in *A. aegypti:* it begins later, involves relatively few individuals, tends to remain at about the same level over a period of days, is not generally increased by shaking the cage, rarely involves the formation of trios, and generally does not involve terminalial contact. If such contact occurs, it is significantly more brief than in heterosexual contacts.

It is proposed that nonheterosexuality in *A. aegypti* occurs when aggressive males become highly excited and are very close to other males which they momentarily perceive as being female.

Nonheterosexual behavior differs critically from homosexuality in that it does not involve a specific choice of the same sex. It differs from bisexuality in that the aggressive partner is not equally attracted to both sexes and generally selects the opposite sex. It seems probable that categorizations of "homosexuality" among many different animals will prove to be examples of nonheterosexual behavior.

I am greatly indebted to Dana R. Pilitt and B. V. Madhukar for raising the insects and for many valuable discussions.

SUMMARY

1. Adult males of *Aedes aegypti* usually avoid each other in flight but sometimes a few of them will chase and capture another flying male. Most of these aggressive males are misaligned and most make no attempt to align or grasp the terminalium of the passive partner. When terminalial clasping occurs, it is always very brief.

2. When 3-day old males are given a choice of contemporaneous or recently-emerged males, they choose the young ones. When given a choice between males and females, males almost invariably choose females.

3. The level of nonheterosexual activity in an all-male cage was measured over a 30 min period over an 11 day span and was first seen when the insects were 36 hr old. From 48 to 276 hr, the level of sexual activity in the cage was found to involve between 10 and 20% of the individuals, thus indicating that 5 to 10% of the animals are aggressive males.

4. All 20 male couples which were isolated from an all-male population flew, yet none of them were chased or captured in a 30 min period. When 40 females were placed in their cage, all the females were quickly chased and copulated with, and none of the males were chased.

5. When a tuning fork of 512 cycles was sounded in an all-male cage of 89 mosquitoes, many males formed dense seething swirls around the fork and as many as 11 couples were formed within the first 32 secs. Most of the individuals in the artifically-induced couplings stood side by side and no attempts were made to orient to or clasp the genitialia of the other individual.

6. Nonheterosexual activity differs from heterosexual behavior in that it appears later, involves relatively few individuals, occurs at a low level for a relatively long period, is not generally increased by shaking the cage, rarely involves trios, and generally does not involve terminalial contact. If such contact is made, it is significantly less than with females.

7. Nonheterosexuality in *A. aegypti* tends to occur when aggressive males become highly excited and are close to other males which they momentarily perceive as being female.

8. Nonheterosexual behavior differs from homosexuality in that it does not involve a specific choice of the same sex. The phenomenon differs from bisexuality in that the aggressive partner is not attracted equally to both sexes and is principally attracted by the opposite sex.

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EARLY DEVELOPMENT OF THE GRUBBY, *MYOXOCEPHALUS AENAEUS* (MITCHILL)[1]

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The grubby, *Myoxocephalus aenaeus* (Mitchill) is a sculpin found from New Jersey north to the Gulf of St. Lawrence (Bigelow and Schroeder, 1953; Needler, 1940; and Jeffers, 1932) and Newfoundland (Ennis, 1969). It inhabits the inshore waters and occurs in water from low tide mark to about 15 fathoms throughout the year (Summer, Osburn and Cole, 1911). Bigelow and Schroeder (1953) report the grubby as common along the shores of the Gulf of Maine and at least one specimen was taken in water as deep as 28 fathoms. Jordan and Evermann (1898) state the grubby is common in seaweeds and Dexter (1944) reports observing it inshore over hard sand and rocky bottoms.

The grubby spawns all winter throughout most of its range. Summer, Osburn and Cole (1911) report finding this true in Buzzards Bay, Massachusetts, and Bigelow and Schroeder (1953) say the same for the Gulf of Maine. Ennis (1969) believes that spawning probably occurs between late fall and early winter around Newfoundland as no egg masses had been found during winter diving. Richards (1959) reports observing 5 to 6 mm larvae in the shallower areas of Long Island Sound from February to April, and Morrow (1951) tells about spawning of the grubby during the winter months in Long Island Sound.

The eggs and larvae of *Myoxocephalus aenaeus* have never been described. Wheatland (1956) says that it is impossible to distinguish larvae of *Myoxocephalus* spp. until the fins are differentiated. Larval descriptions have been given for *M. scorpius* by Ehrenbalm (1904) but nothing is available for *M. aenaeus* except Perlmutter's (1939) sketch of a 6 mm larvae. The objective of this paper is to describe the early development of *M. aenaeus* and to contribute information on its early life history.

MATERIALS AND METHODS

Adult grubbys were collected on various dates from December through March in the Mystic River at Groton, Connecticut, with a 50-foot, knotless-nylon seine and were taken to the laboratory where they were maintained in continuous-flow aquariums. Eggs were artificially fertilized by stripping male and female grubbys into 21 cm fingerbowls containing sea water which had been filtered through 0.45 μ filters. Fingerbowls were kept in a water bath of running sea water so that eggs were fertilized at approximately the ambient temperature and remained there dur-

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TABLE I

Summary of the successful artificial fertilizations of Myoxocephalus aenaeus *eggs.*

Egg color	Number parents	Date of fertilization	Days to first hatch	Days when most hatched	Range in days of hatching period	Average temperature (°C)	Temperature range (°C)
Red	2	Dec. 23	42	42	42	4.6	3.0–7.0
Green	2	Jan. 11	42	50–53	42–57	5.3	3.0–8.0
White and red	18	Feb. 12	34	40–44	34–47	5.8	4.5–7.0
White and red	9	Feb. 18	36	42–46	40–53	6.0	4.4–8.0

ing the entire developmental period. The water in the fingerbowls was first changed to remove excess sperm after the eggs were water hardened. From that point on, the water was changed about every three days. Initially, all fingerbowls were aerated. Later, half the fingerbowls were not aerated when it became apparent that aeration was not absolutely necessary.

Eggs and larvae in the fingerbowls were observed under a dissecting microscope and developmental times were noted. Eggs were fertilized on four different occasions (Table I). Large mortalities occurred in rearing to the young stage, and multiple fertilizations were necessary to have adequate samples for detailed descriptions of all stages. The drawings and descriptions are from all fertilizations. Samples for drawings were preserved in 5% neutralized formalin. All drawings were made with the aid of a camera lucida.

Larvae were first fed Liqua Fry and later brine shrimp nauplii.

RESULTS AND DISCUSSION

Field work began on 14 December 1964 when ripe males and females were collected. Ripe fish were found on the six collecting days in December, the two in January, the four in February and the two in March. No collecting was done in April, and on 19 May all seven females collected were spent. Water temperatures varied from 4° C on 14 December to 1° C in late February and early March. The water temperature was 3° C when the last ripe fish were caught on 30 March. In this area, the grubby spawns over a four-month period from December through March.

The egg of the grubby is spherical, transparent and adhesive. Small clumps of eggs have been found attached to many different substrates including vegetation, shells, stones, bryozoans and wooden traps. The color of the eggs is variable but is consistent within a single female. The eggs are light green, red, white and sometimes yellow in color. Morrow (1951) reports similar variations in color with the eggs of *Myoxocephalus octodecemspinosus.*

Table I summarizes the artifical fertilizations and incubation periods of eggs during this study.

Water-hardened eggs were spherical with an average diameter of 1.58 mm (range, 1.5–1.7 mm). The average yolk diameter was 1.46 mm (range, 1.3–1.6 mm). The egg capsule was thin and transparent with a smooth surface. The yolk had two large oil globules with an average diameter of 0.2 mm and a few smaller globules scattered throughout. The perivitelline space was narrow, about one-sixth of the egg radius (Fig. 1A).

2-cell stage: the first cleavage was meroblastic and was observed about 8½ hours after fertilization; the majority of the eggs were in this stage at 10½ hours. The two blastomeres were equal in size, raised and separated. The average egg diameter was 1.59 mm (range, 1.5–1.7 mm) and the average yolk diameter was 1.39 mm (range, 1.1–1.6 mm). The yolk was slightly corrugated with two to four large oil globules near the positive pole of the egg. The perivitelline space was one-fourth of the egg radius (Fig. 1B).

4-cell stage: the first 4-cell stage was found after 10 hours, and the majority of the eggs were in this stage at 15 hours. The four flattened blastomeres were approximately equal in size (Fig. 1C).

8-cell stage: this stage was first noticed at 16 hours and the majority of the eggs were in this stage at 20 hours (Fig. 1D).

16-cell stage: most eggs were in the 16-cell stage between 21 and 22 hours. The cells were of irregular shape, raised and in a single layer (Fig. 1E).

32-cell stage: the majority of the eggs were in this stage at 27 hours. The central cells were in a two-cell layer while the peripheral cells were in a single layer (Fig. 1F).

Multicellular stage: eggs classified as in this stage were first observed at 33 hours, and all were in this stage at 96 hours. The blastodisc remained approximately the same size during this period of multiplication of cells (Figs. 2A and 2B).

Blastula stage: blastocoele was evident and was flattened out over the yolk. Most eggs reached this stage between 98 and 196 hours (Fig. 2C).

Gastrula stage: the germ ring and the embryonic shield were evident. The germ ring was first evident at about 174 hours (7.25 days) after fertilization. The primitive streak was faint (Fig. 2D).

Early embryonic stages: the neural groove extended about one-half way around the yolk. The embryo developed visible cephalic and caudal swellings. The optic vesicles were forming and a few small oil droplets were concentrated in the caudal region (Figs. 2E and 2F).

Middle embryonic stages: embryo was elongated more than one-half way around the yolk and nine somites were evident near the caudal region. The lens of the eye was thickened and was bulging out of the optic cup. There were one to two large oil globules near the negative pole of the egg (Fig. 3A).

The embryo was in the tail-free stage. Kupffer's vesicle was evident. The divisions of the brain were differentiated. The olfactory placode, pectoral bud, auditory vesicle and notochord were present. The first movement was noted within the egg (Fig. 3B).

Late embryonic stages: retinal pigment was first seen and the lens of the eye was pronounced. Finfold was evident. Heartbeat was first seen. About 21 somites were seen over one-half of the body. Two large oil globules were near the cephalic region and a few smaller ones near the caudal region (Fig. 3C).

There was a continuing increase in the size of the embryo with a gradual decrease in the size of the yolk sac (Fig. 3D). On the lateral flexion, the tip of the tail passes over the hindbrain. The heart was situated in the frontal concavity of the yolk sac. The otoliths were visible and the pectoral fins curved. The rudiment of the lower jaw was present and the head more flattened. The embryo exhibited much twitching at this stage.

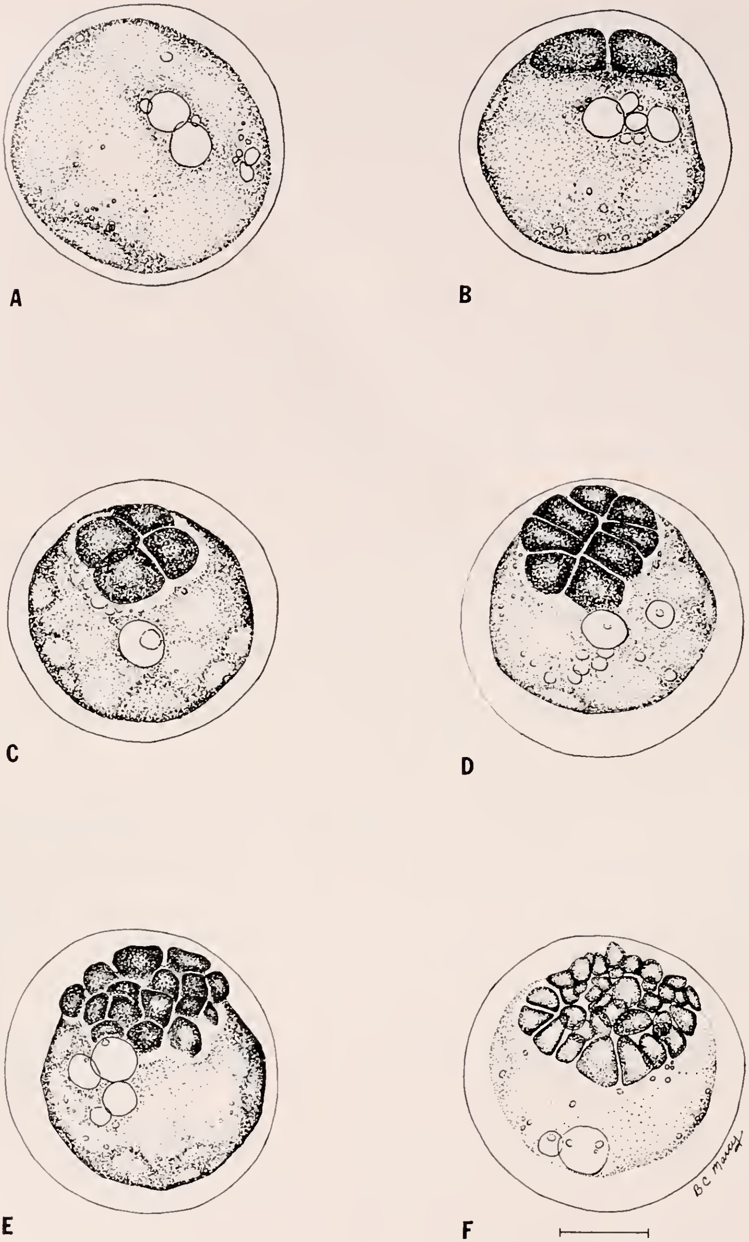

FIGURE 1. Early development of the eggs of the grubby, *Myoxocephalus aenaeus,* artificially propagated in the laboratory: A) unfertilized water-hardened egg, 1.58 mm; B) fertilized egg, 1.59 mm, two-cell stage (12 hr, 20 min); C) 4-cell stage, 1.60 mm (15 hr, 50 min); D) 8-cell stage, 1.61 mm (16 hr, 55 min); E) 16-cell stage, 1.54 mm (21 hr, 10 min); F) 32-cell stage, 1.60 mm (27 hr, 40 min), scale = 0.5 mm. Measurements refer to mean egg capsule diameters.

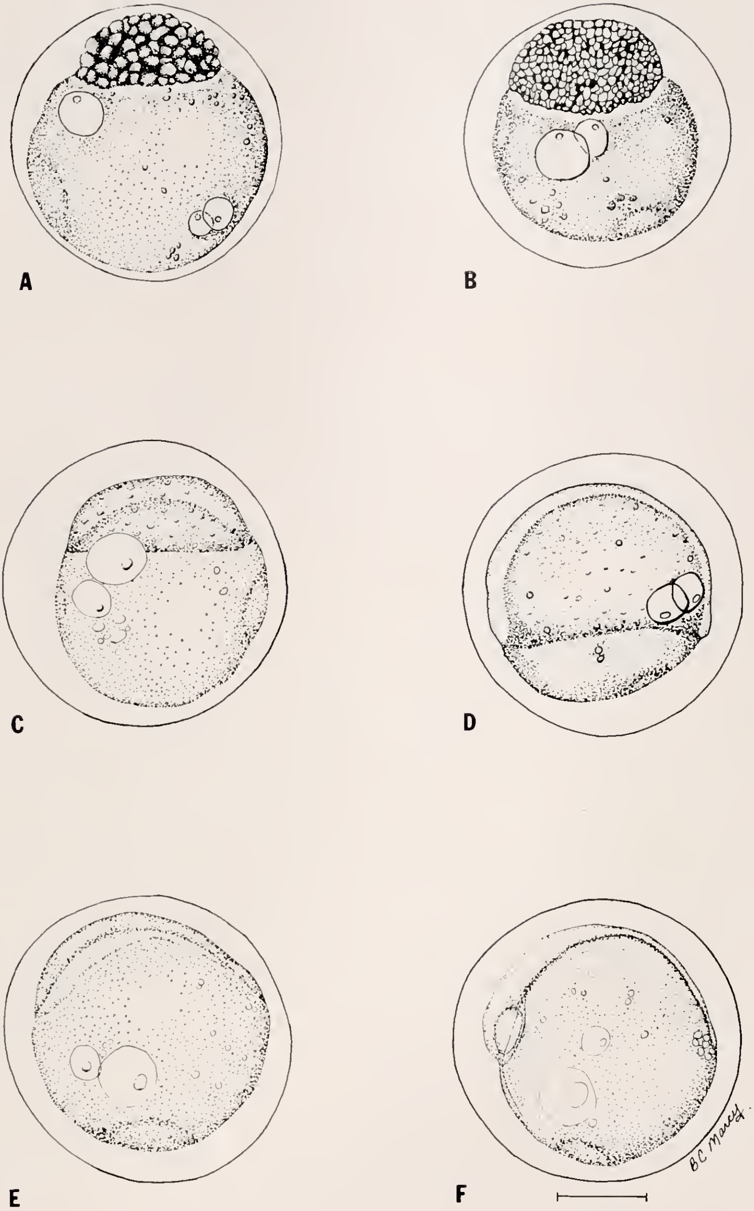

FIGURE 2. Development of the eggs of the grubby, *Myoxocephalus aenaeus*, artificially propagated in the laboratory: A-B) 64-cell to multicellular stage, 1.60 and 1.57 mm (33–98 hr); C) blastula stage, 1.53 mm (196 hr—8 days); D) gastrula stage, 1.53 mm (196 hr—8 days); E-F) early embroynic stages, 1.60 mm (189–246 hr—7.8 to 10.3 days), scale = 0.5 mm. Measurements refer to mean egg capsule diameters.

At this stage the tail was now curved around the head and the pectoral fins were raised above the yolk sac (Fig. 3E). The first evidence of pigmentation was a sprinkling of contracted melanophores on the dorsal side of the head near the optic vesicles. Stellate melanophores were next evident between the yolk sac and the body, posterior to the pectoral fins. The anal vent was now apparent and the eyes appeared to have lost the deep fissure.

The rays in the caudal fin were faintly seen (Fig. 3F). The lower jaw was more developed and the mouth was open. The melanophores on the surface of the yolk were expanding with a band running diagonally from the anal vent to below the head. Most embryos showed seven to ten melanophores between the auditory vesicles on the dorsal surface. The circulatory system was noticeable along the length of the tail. There was much twitching and head extension of the embryo.

Prehatching stage: the embryo occupied almost the entire egg capsule and was able to make full revolutions within the egg. The tail was wrapped almost completely around the body. The pectoral fins occasionally showed fluttering movements between long periods of quiescence. There was now a single large oil globule near the head. The margin of the operculum appeared well defined. Evenly spaced melanophores extended from behind the vent along the ventral line of the tail. Dense melanophores were present on the dorsal surface of the yolk sac (Fig. 4A).

Most prolarvae hatched between 40 and 44 days (Fig. 4B). Most emerged from the egg head first but a few were observed to emerge tail first. The prolarvae ranged between 4.7 and 6.3 mm TL (mean, 5.4 mm). The yolk sac measured 0.9 mm. As in the egg stages, the large oil globule contained a small bubble. The eye was darkly pigmented. Additional stellate chromatophores were present and scattered in two bands over the ventral yolk sac with a large concentration along the intestine and a series of 20 to 24 spots along the lower ventral line of the tail. The larvae had 32 myomeres. The dorsal finfold was continuous with a lunate caudal portion. The incipient rays of the caudal fin were faintly seen and the lines of ossification were present along the notochord. The auditory vesicles bulged above the eye and the mouth was still not well developed.

The yolk sac and oil globule were absorbed about five days after hatching (6.1 mm TL) (Fig. 4C). Large dark contracted chromatophores were present over the gut area with 30 to 34 spots along the lower ventral line of the tail. A few chromatophores extended onto the finfold in the caudal region. There were large expanded chromatophores at the base of the pectoral fin and a few scattered behind the auditory vesicles. The mouth was fully developed and functional. Larvae now approached the brine shrimp nauplii, coiled their tails, struck and settled to the bottom for a short rest period. Two head spines were developed on the dorsal bulge of the auditory vesicles and four were seen on each side of the head along the operculum. The postlarvae had 32 myomeres and the finfold was smooth and continuous.

In a 14-day-old larva (6.8 mm), the finfold was more ragged and the first incipient rays of the dorsal fin were present. Chromatophores were present along the developing caudal and pectoral fins. The latter had prominent rays. Four gill arches were noticeable. The chromatophores along the intestine were dense, contracted and easily observed with the naked eye. Brown to orange stellate

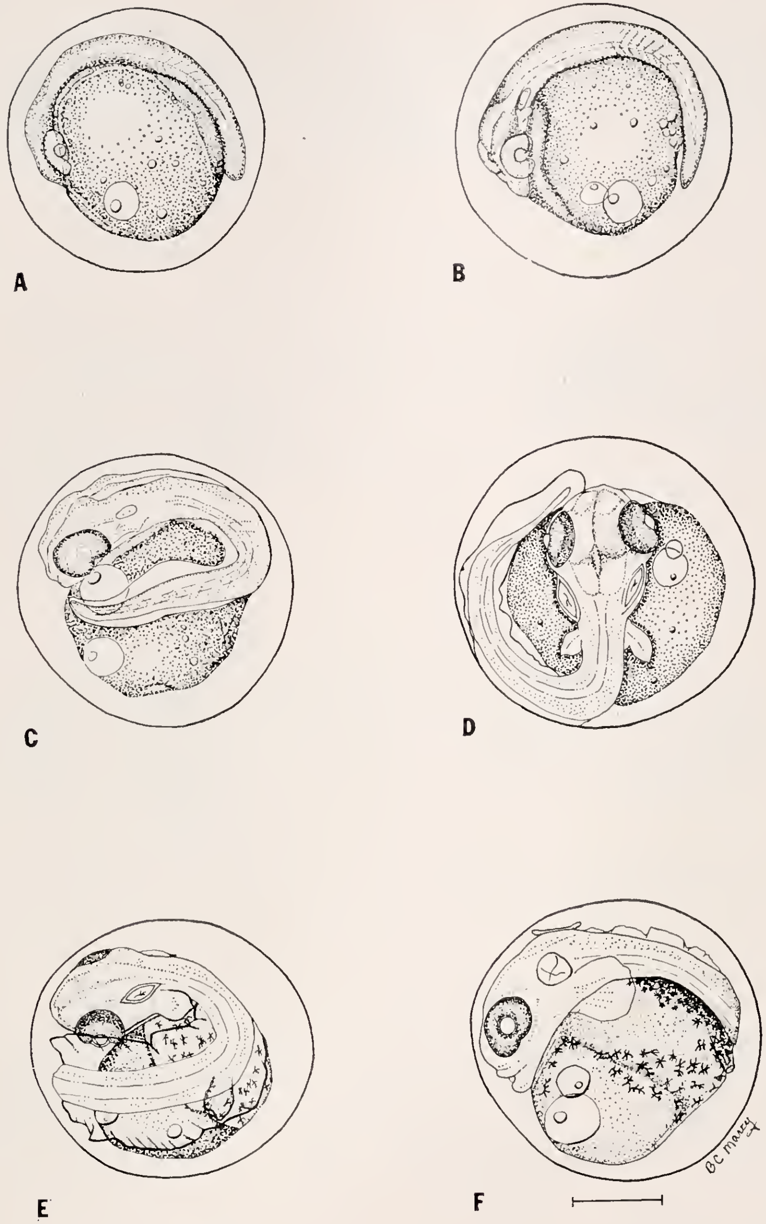

FIGURE 3. Later development of the eggs of the grubby, *Myoxocephalus aenaeus,* artificially propagated in the laboratory: A) developing embryo, 1.60 mm (325 hr—13.5 days); B) developing embryo, 1.57 mm (350 hr—14.6 days); C) developing embryo, 1.60 mm (415 hr—17.3 days); D) developing embryo, 1.67 mm (552 hr—23 days); E) late developing embryo, 1.60 mm (605 hr—25.2 days); F) late developing embryo, 1.60 mm (725 hr—30.2 days), scale = 0.5 mm. Measurements refer to mean egg capsule diameters.

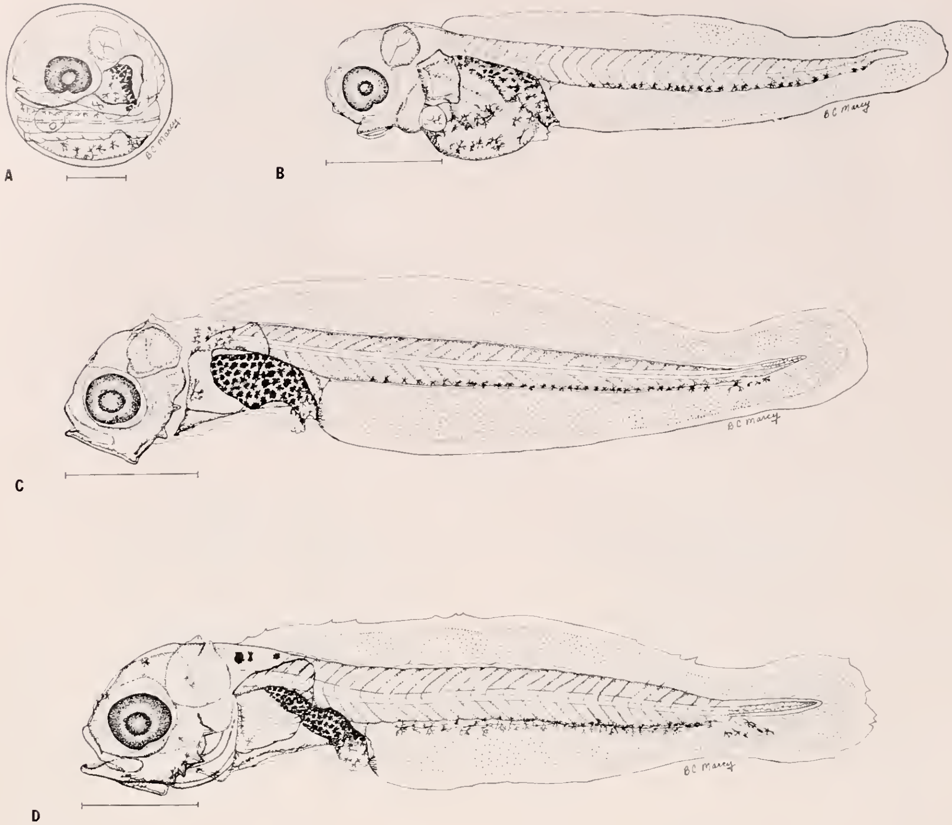

FIGURE 4. Pre-hatch embryo, prolarval and early postlarval stages of the grubby, *Myoxocephalus aenaeus,* artificially propagated in the laboratory: A) pre-hatch, well developed embryo, 1.65 mm (960–1,056 hr, 40–44 days), scale = 0.5 mm; B) newly hatched prolarva 5.4 mm TL, scale = 1 mm; C) postlarva, 6.1 mm TL (5 days old), scale = 1 mm; D) postlarva, 6.8 mm TL (14 days old), scale = 1 mm. Measurements refer to mean egg capsule diameter and mean total length of larvae.

chromatophores were evident above the pectoral fin and along the top of the head, the cleithrum, and the preopercle at the base of each spine (Fig. 4D).

Pectoral fins were elongating on the dorsal side and an anlage of the dorsal spines was developing along the dorsal line of the tail (Fig. 5A). The caudal fin rays were distinctly bifurcate. Five spines were now evident along the operculum. Two spines were present on each side of the head above and joined to the auditory vesicles. The chromatophores along the the stomach and intestine were almost continuous with fewer toward the vent. Large stellate chromatophores were more prominent on the head with dark vertical barring appearing posterior to the auditory vesicles.

The dorsal head spine appeared as two distinct spines on each side (Fig. 5B). The anal fin rays were developing with stellate chromatophores located along the ventral line of the tail. The ventral fin was now evident and the pectoral fin was

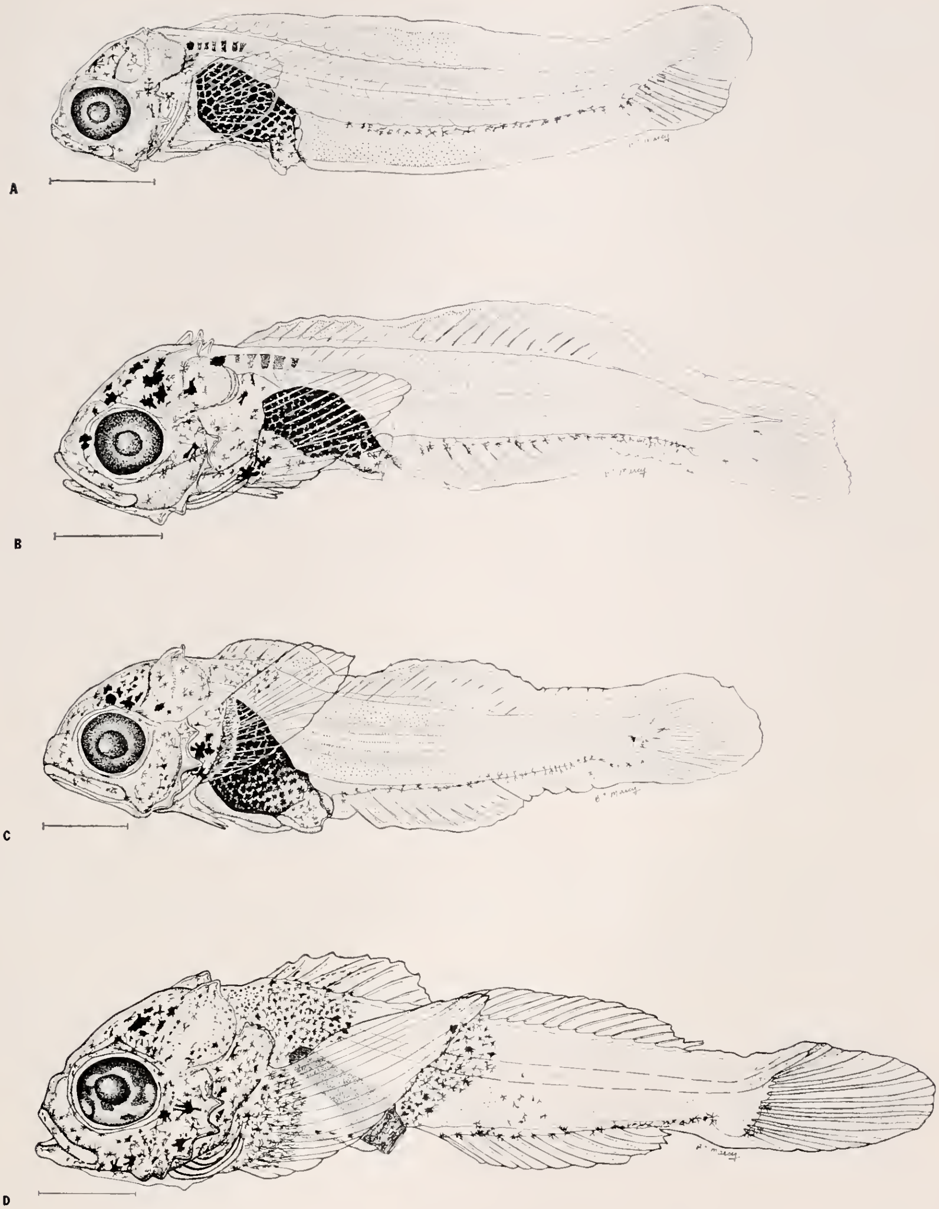

FIGURE 5. Later postlarval and young stages of the grubby, *Myoxocephalus aenaeus*, artificially propagated in the laboratory: A) late postlarva, 6.8 mm TL (22 days old), scale = 1 mm; B) late postlarva, 7.5 mm TL (30 days old), scale = 1 mm; C) postlarva prior to transformation to young, 8.5 mm TL (45 days old), scale = 1 mm; D) transformed young with adult characteristics, 9.2 mm TL (55 days after hatching), scale = 1 mm. Measurements refer to mean total length of larvae and young.

becoming fan-like. The finfold was continous but irregular. Large dark chromatophores appeared above the eye, at the base of the pectoral fin and below the dorsal fin.

In a 45-day-old larva (8.5 mm), the fins, except for the dorsal, were still incomplete. The pectoral fin extended past the dorsal fin. The two dorsal head spines on each side overlapped each other and appeared as one large structure. The dark pigment bars above the eye had transformed into stellate chromatophores extending up into the first dorsal fin, and there was further pigmentation in the head area.

The grubby is transformed from the larval stage at approximately 9 mm TL (Fig. 5D), approximately 55 days after hatching. The first dorsal fin had nine spines and was shorter (front to rear) than the second dorsal of 14 rays. The anal fin had 11 rays. The urostyle had turned dorsally. There were now six spines along the operculum and the two head spines noted in the previous two stages had become one large spine covered by tissue. There was development of a pair of spines between the nostrils. The head was covered with large brown stellate chromatophores. The intestine and stomach appeared solid dark brown. Bands of chromatophores were forming diagonally from ventral to dorsal surfaces along the body. Pigmentation along the ventral line of the tail was still regular.

Young were raised in the laboratory until 108 days after hatching (12.8–21.0 mm TL). Growth was most rapid between 55 and 108 days. The head became more flattened and the snout more pointed with head spines becoming larger and well defined. The body became covered with dark shading or irregular barrings as the young took on typical adult coloration.

All original drawings were made by B. C. Marcy, Jr.

SUMMARY

1. The grubby spawns over the four-month period of December through March in the Mystic River, Connecticut. The spherical, adhesive eggs are light green, red, white, and sometimes yellow in color and are found in small clumps attached to many different substrates.

2. Eggs were artificially fertilized on four occasions over a two-month period. The average water temperatures during development ranged from 4.6 to 6.0° C. The first cleavage occurred between $8\frac{1}{2}$ and $10\frac{1}{2}$ hours after fertilization and progressed to the multicellular stage at 33 to 96 hours. Gastrulation was first observed at about 174 hours, and the total developmental period varied from 34 to 57 days with most hatching between 40 and 44 days.

3. Prolarvae ranged between 4.7 and 6.3 mm TL, and the yolk sacs were absorbed in about five days. All fins were developed in approximately 55 days. During the period between 55 and 108 days, pigmentation was completed and the young took on the typical adult coloration.

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AUTOGENOUS EGG PRODUCTION IN THE SALT-MARSH MOSQUITO, *AEDES TAENIORHYNCHUS*

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Female mosquitoes are noted for their blood-feeding activities. Usually a blood meal is a prerequisite to egg maturation, yet more than 40 species of mosquitoes can produce at least the initial egg batch on a blood-free diet (Vinogradova, 1965; Aslamkhan and Laven, 1970). This capacity is called autogeny, while the term anautogeny applies to those cases where blood is normally always necessary for egg development. The females of some species appear to be uniformly autogenous, whereas others have both autogenous and anautogenous populations (Spielman, 1971). Autogeny also occurs in other hematophagous Diptera and it is characteristic of several species which inhabit the tidelands. Salt-marsh tabanids, such as the deer flies, *Chrysops fuliginosus* and *C. atlanticus,* and the horsefly, *Tabanus nigrovittatus*, normally do not bite until they have laid their first batch of eggs (Anderson, 1971; Rockel, 1969; Bosler and Hansens, 1974). Similar feeding patterns have been observed in certain salt-marsh sandflies (Linley and Davies, 1971).

Autogeny was discovered in *Aedes taeniorhynchus* by Lea and Lum (1959) in some Florida populations. More recently, O'Meara and Evans (1973) reported a clinal variation in which both the frequency and the fecundity of autogenous females increased along a North to South gradient. Geographical variation in autogeny has a genetic component that is monofactorial in some species and polygenic in others (O'Meara and Craig, 1969; Rioux, Croset, Gabinaud, Papierok and Belmonte, 1973; Spielman, 1957; Laven 1967). Although populations of *A. taeniorhynchus* differ in their genetic potential for autogeny, genetic factors responsible for these differences have remained poorly defined. Similarly, little is known about the relative influence of specific environmental factors on the occurrence of autogeny in field populations of *A. taeniorhynchus*. The absence of suitable hosts and the inhibition of host seeking by harsh climatic conditions appear to be major factors selecting for autogeny in species inhabiting the variable and at times severe climatic regions of the arctic and temperate zones (Corbet, 1964, 1967; Downes, 1965; Smith and Brust, 1970). Nevertheless, for many species the distribution of autogeny cannot be readily attributed to these or other specific ecological factors (O'Meara and Craig, 1970; Moore, 1963).

In most mosquito species, autogenous females do not blood feed until the initial egg batch is laid (Spielman, 1971; Hudson, 1970). However, ovarian development is more flexible in *A. taeniorhynchus* and autogenous females will readily blood-feed during the first and second day following emergence. This blood-feeding will increase the size of the initial egg batch. Larval nutrition can influence the expression of autogeny, both the frequency and the fecundity of autogenous females being reduced under poor nutritional conditions (Lea, 1964).

The present study examines three aspects of autogenous reproduction in *A. taeniorhynchus*. First, additional information is provided on the occurrence of autogeny throughout the range of this species. Secondly, the genetic regulation of fecundity in autogenous females is investigated with crossing experiments. And finally, for two field populations, comparisons are made between the patterns and rates of blood-feeding and the autogenous potential.

MATERIALS AND METHODS

The laboratory strains used originated from four different geographical areas: New Jersey (Rutgers), Louisiana (Lake Charles), Florida (North Key Largo), and the Cayman Islands (Grand Cayman). The Rutgers, Lake Charles, and the North Key Largo (NKL) strains have been maintained in the laboratory for 10 or more years. The Cayman strain was started in 1971. In addition to colonized material, eggs of wild *A. taeniorhynchus* were obtained from sod and leaf litter or from females taken in biting or aspirator collections. Mosquito rearing and colony maintenance were performed in an insectary with a controlled environment. The temperature was maintained at 26 ± 1.5° C and the relative humidity at 80 ± 15%, while the daily photoperiod was 14.5 hours. The larval and adult diets were the same as those used by O'Meara and Evans (1973).

For laboratory-reared mosquitoes, ovarian examinations were performed on females only after they were at least 5 days old. Only development to Stage V was classified as autogenous. Both an exochorion and a micropyle apparatus are present at this stage of oocyte development. Fecundity was measured by counting both laid eggs and Stage V "eggs" within the ovary of each female.

At Flamingo and Big Pine Key, Florida, adult mosquitoes were captured with a 12 volt battery-powered aspirator. This device has a cylindrical aluminum frame, 14 inches in diameter and 5 ft in length and a rigid transparent intake. The suction provided by a rear mounted fan pulls mosquitoes into a screen cone which has a net bag at the apex. With the battery contained in a backpack, the aspirator could be operated in the mangrove swamp where it would be difficult to use a trap mounted on a vehicle (Bidlingmayer and Edman, 1967). The power aspirator was used during the daytime when salt-marsh mosquitoes are normally resting on the ground litter or vegetation. Net bags containing mosquito collections were transported in an ice chest from the field to the laboratory where they were stored at −25° C until they could be sorted, identified and tested.

The host-blood sources of engorged females in aspirator collections from Flamingo and Big Pine Key were determined serologically by capillary precipitin tests (Tempelis and Lofy, 1963). Extracts of individual blood meals were first tested with two broadly-reactive screening antisera to establish whether blood meals were mammalian or avian in origin. Reddish extracts failing to react with either antiserum were tested with a third screening antiserum, reactive with amphibian and reptilian bloods. After screening tests, blood-meal extracts were further tested with appropriate antisera specific for different hosts or closely-related host groups within each class of vertebrates. Methods for the preparation of all antisera used as well as the mechanics of the testing are contained in previous publications (Edman, 1971; Edman, Webber and Kale, 1972a).

TABLE I

The occurrence and the fecundity of autogenous females in laboratory strains and field populations of Aedes taeniorhynchus.

Population	Females		
	Number examined	% autogenous	Eggs/autogenous ♀ (Mean ± SE)
(Laboratory colonies)			
Rutgers	542	4.1	23.1 ± 0.6
Lake Charles	277	11.2	28.5 ± 1.6
NKL	102	83.3	71.8 ± 1.8
Cayman	99	83.8	63.1 ± 1.1
(Field populations)*			
Panama, Canal Zone	201	0.5	7.0
Lake Charles, Louisiana	107	1.0	25.0
Savannah, Georgia	112	8.0	48.7 ± 6.2
Fernandina, Florida**	2146	21.6	39.6 ± 0.6
Allenhurst, Florida	307	27.4	45.2 ± 1.8
Oak Hill, Florida**	2086	57.0	45.7 ± 0.4
Sebastian, Florida	148	57.5	52.4 ± 1.6
Jack Island, Florida**	1979	60.7	45.5 ± 0.4
Big Pine Key, Florida	199	74.0	55.4 ± 1.5
Little Duck Key, Florida	200	75.0	63.6 ± 1.2
San Juan, Puerto Rico	43	83.7	48.5 ± 1.0
Grand Cayman Island, British West Indies	337	91.4	57.4 ± 0.6
Flamingo, Florida**	1948	94.4	66.9 ± 0.3

* F_1 progeny of wild-caught females.

** Based on two or more separate field collections.

RESULTS

Every population examined contained a few autogenous females (Table I). Among the laboratory strains, the levels of autogeny were low in Lake Charles and Rutgers and they were high in Cayman and North Key Largo (NKL). For field populations the frequency of autogeny varied over a wide range. In general, the rates were higher in populations from Southern Florida and the Caribbean region than they were in those that originated from northern Florida, Louisiana, Georgia and New Jersey. A major exception to this pattern was the Panama Canal Zone population which had the lowest rate of any group. Geographical variation was also noted in the mean egg production of autogenous females. The mean fecundity was often less than 30 eggs/autogenous female in those populations where the occurrence of autogeny was rare or infrequent. In contrast, significantly higher levels of fecundity were found in all populations which contained mostly autogenous females (Table I).

Inadequate larval and adult nutrition reduces or eliminates autogenous egg production. But since we used a single highly nutritious diet, the observed differences in the occurrence and the expression of autogeny must be genic. We examined the offspring of crosses involving the Rutgers and the Cayman strain

TABLE II

Autogenous egg production in the progeny of crosses involving the Cayman and Rutgers strains.

Cross		Autogenous progeny	
♀	♂	Number examined	Mean number of eggs per autogenous ♀*
Cayman	Cayman	201	62.9 ± 0.8
Rutgers	Rutgers	157	27.0 ± 0.6
Cayman	Rutgers	150	51.7 ± 0.9
Rutgers	Cayman	159	52.1 ± 0.8
Cay/Rut	Cay/Rut	163	42.7 ± 1.1
Rut/Cay	Rut/Cay	446	44.1 ± 0.6
Cayman	Rut/Cay	224	54.2 ± 0.7
Rut/Cay	Cayman	200	52.1 ± 0.9
Rutgers	Rut/Cay	470	33.2 ± 0.4
Rut/Cay	Rutgers	193	36.4 ± 0.9

* Mean ± SE.

in an attempt to obtain additional information on the genetic factors regulating autogenous egg production. The autogenous Cayman females develop more than twice as many eggs as the Rutgers females. The egg production of F_1 and F_2 progeny was intermediate, although the F_1 hybrid more closely approached the level found in the Cayman females. When F_1 hybrids were backcrossed to the Cayman strain the fecundity of the progeny was essentially the same as that found in F_1 hybrids, whereas backcrosses to the Rutgers strain gave females which had a significant reduction in egg production (Table II).

To determine if autogenous and anautogenous females could occur in the same reproductive population we examined the offspring of individual, wild females. Five field sites were tested and at each site *A. taeniorhynchus* females were collected as they attempted to take a human blood meal. These wild females usually produced F_1 progenies containing autogenous and anautogenous mosquitoes (Table III). All but 2 of 103 females from Jack Island and Oak Hill, Florida

TABLE III

The occurrence of autogeny in the F_1 progenies of field collected Aedes taeniorhynchus *females.*

Collecting site	F_1 progenies			
	Number with autogenous females only	Number with anautogenous females only	Number with both types of females	Total number examined
Fernandina	0	11	41	52
Oak Hill	0	0	51	51
Jack Island	2	0	50	52
Flamingo	21	0	29	50
Cayman	2	0	7	9

produced both kinds of offspring. The majority of the wild females from Flamingo, Florida and Cayman Islands gave F_1 progenies with both types of females. Of the 5 field sites examined, only Fernandina females produced some uniformly anautogenous progenies. Nevertheless, even for this site mixed progenies predominated. The frequency of autogeny in the progeny of individual females was highly variable. However, it tended to reflect the overall rates for each population. For example, the level of autogeny did not exceed 20% in most progenies of individual Fernandina females. In contrast, this rate was often greater than 90% in both Cayman and Flamingo progenies.

If the lack or scarcity of suitable hosts is a major factor selecting for genotypes for autogeny in *A. taeniorhynchus,* we would expect such a condition to also influence the mosquito's blood-feeding activities. Without an adequate supply of suitable hosts, engorgement rates would be depressed and the relative frequency of "blood-thirsty" females would be increased. It is difficult to predict how host scarcity would affect the utilization of specific hosts. Previous studies have shown that *A. taeniorhynchus* feeds primarily on certain mammals (Edman, 1971). However, if these mammals are not readily available, then there might be an increase in feeding on other types of vertebrates such as birds, reptiles, and amphibians.

Our evaluation of the blood-feeding patterns and rates was based on the examination of specimens which had been taken with the power aspirator. At both Flamingo and Big Pine Key, Florida, six monthly collections were made during the summer and fall seasons. Resting adults were found to be most common in areas dominated by black mangrove and buttonwood trees. We, therefore, confined most of our sampling to these habitats. Several thousand mosquitoes were collected with the power aspirator. Freezing these specimens proved to be not only an effective method for storage but it also facilitated the work of sorting and classifying the mosquitoes. Engorged and gravid females could be readily identified in frozen specimens because the blood meal or the eggs were clearly visible through the transparent, membranous portions of the abdomen.

All *A. taeniorhynchus* females from the aspirator collections were separated into engorged and non-engorged groups. The latter group was subdivided into gravid and nongravid females. Engorged females can usually be recognized for at least 72 hours after taking a blood meal. But once the meal has been completely digested it is rather difficult to find any direct evidence of a prior blood meal. Egg development initiated by a blood meal is rapid and the completion of egg maturation (Stage V) normally occurs in 4 to 5 days. We considered females to be gravid only if they contained ovaries with development to Stage V. At Big Pine Key the engorgement rate was nearly 3 times the rate found at Flamingo. Gravid females were also more frequent at Big Pine Key (Table IV). Still, most females from both collecting sites were neither gravid nor engorged. We attempted to determine what proportion of these females were at a stage where they would actively seek a blood source. Young adults, less than one day old, are reluctant blood-feeders. Similar behavior is often displayed by older females who have ovarian follicles at Stage III or IV. *Aedes taeniorhynchus* females do not become inseminated until they are at least 1 day old (Edman, Haeger, Bidlingmayer, Dow, Nayar and Provost, 1972). Thus the presence of sperm in the spermatheca is clear evidence that the mosquito is old enough to be a blood-

TABLE IV

The frequency of engorged, gravid and non-gravid females in power aspirator collections.

Collecting site	Females			
	N	% engorged	% non-engorged	
			gravid	non-gravid
Flamingo	13,612	8.1	8.4	83.4
Big Pine Key	6,421	20.9	11.5	67.6

feeder. For each monthly aspirator collection we examined the spermatheca of 50 females. Nearly all of these females had sperm in their spermatheca. Moreover, when we examined the ovaries of 100 or more females from each aspirator collection, only a very small percentage of these non-engorged, non-gravid females had their ovaries at developmental Stages III or IV. The vast majority of these females had their ovaries at Stages I or II (Table V). Therefore, approximately 80% of all females from the Flamingo aspirator collections could be called "bloodthirsty" mosquitoes. In contrast, only about 60% of the Big Pine Key females were in this category.

The results of precipitin tests on engorged mosquitoes are presented in Table VI. The unidentified avian and unidentified mammalian groups represent blood meals which reacted with the appropriate class-specific screening antiserum but were probably too digested for more specific characterizations. A few may represent feeding on hosts which were not included in the test. The blood contained in the guts of engorged females had been obtained from a variety of vertebrate hosts. Yet, overall most mosquitoes had fed on mammals. Nearly 90% of the identified blood meals in Flamingo females came from mammals. For Big Pine Key females, mammal feedings represented about 80% of the total. Rabbits (*Sylvilagus palustris*) were certainly the predominant host for *A. taeniorhynchus* at Flamingo. Among the identified mammalian blood meals, 87% were rabbit's blood. Most engorged mosquitoes from Big Pine Key contained the blood of another mammalian group, the ruminants. The only member of the suborder, Ruminantia, known to be present on this island is the Key deer (*Odocoileus virginianus clavium*). When ruminant blood meals were further characterized by examin-

TABLE V

The degree of ovarian development in wild females that were neither engorged nor gravid.

Collecting site	Number examined	% females at stage		
		I or II	III	IV
Flamingo	1108	93.3	6.4	0.3
Big Pine Key	2171	94.1	5.4	0.4

TABLE VI

Host blood sources of A. taeniorhynchus *collected with the power aspirator at two south Florida localities.*

Blood source	Collection site Big Pine Key	 Flamingo
Rabbit	7 (2)*	373 (87)*
Ruminant	379 (96)	0 —
Raccoon	9 (2)	14 (3)
Rodent	0 —	16 (4)
Human	1 (<1)	16 (4)
Other**	0 —	10 (2)
Unidentified mammal	18	29
Total mammal (%)	(79)	(90)
Ciconiiformes	102	21
Passeriformes	0	1
Unidentified avian	6	28
Total avian (%)	(21)	(10)
Total number identified/number tested	522/540	511***/563

* Percent of all identified mammalian hosts.

** Included feedings on opossums and armadillos.

*** Totals for the Flamingo site include one amphibian and two reptilian feedings.

ing their hemoglobin crystals (Washino and Else, 1972), all positive tests indicated the presence of deer blood. Thus most if not all of the ruminant blood meals, which represented 96% of all mammalian feedings by Big Pine Key females, probably came from Key deer.

Every time we sampled a site with the power aspirator, we made a follow-up biting collection the same day. The capacity for autogeny was measured in the F_1 progeny of these wild caught females. Both the frequency and the fecundity of autogenous females were consistently greater at Flamingo than they were at Big Pine Key. From a sample of 1,768 F_1 Flamingo females 86.9% were autogenous and they had a mean autogenous fecundity of 65 eggs per autogenous female. In contrast, only 65.5% (N = 1,697) of the F_1 Big Pine Key females were autogenous and they had a mean autogenous fecundity of 51 eggs per female.

DISCUSSION

When O'Meara and Evans (1973) examined the seasonal and geographical distribution of autogeny in peninsular Florida, they found a progressive increase in the frequency of autogeny from grassy salt marsh to mangrove sites. No evidence was found for seasonal variation in autogenous genotypes. The findings of the present study also show that autogeny tends to be less frequent in the grassy marshes of the temperate zone than it is in mangrove sites of the subtropics and tropics. However, high rates of autogeny are not always associated with tropical populations of *A. taeniorhynchus*, *e.g.*, the Panama Canal Zone population (Table I).

For other mosquito species geographical variations in autogeny have different patterns. In the North American rock pool mosquito, *Aedes atropalpus*, there are four subspecies. Populations of the type form, which occupy the northern and northeastern portion of this species' range, are uniformly autogenous, whereas the three southern subspecies are usually completely anautogenous (O'Meara and Craig, 1970). Autogeny in *Culex pipiens* is often associated with the range of the temperate zone subspecies, *C. p. pipiens,* but it is normally absent in the tropical subspecies, *C. p. quinquefasciatus* (Spielman, 1971). Rioux, Croset, Gabinaud, Papierok and Belmonte (1973) found a totally autogenous population of *Aedes detritus* in France and a completely anautogenous population in Tunisia. Strains of *A. togoi* vary from wholly autogenous to partially autogenous to anautogenous (Laurence, 1964; Thomas and Leng, 1972). Clearly the patterns of geographical variation among autogenous mosquitoes are quite diverse.

Spielman (1964) found sympatric autogenous and anautogenous populations of the *Culex pipiens* group to be reproductively isolated in nature. Ellis and Brust (1973) have recently delineated sibling species in the *Aedes communis* aggregate. One species, *A. churchillensis,* is uniformly autogenous, while another sibling species, *A. communis* is normally anautogenous. There are both allopatric and sympatric populations of these sibling species. A different situation exists in *A. taeniorhynchus.* Wild caught females usually produce progeny containing both autogenous and anautogenous females (Table III). The two types of females apparently can and often do occur in the same reproductive population.

Moore (1963) and Spadoni, Nelson and Reeves (1974) have found seasonal variations in both the frequency and the fecundity of autogenous females. It is not known if these seasonal patterns involve changes in the frequency of autogenous genotypes. O'Meara (1972) has uncovered a polygenic system regulating the fecundity of autogenous females in *Aedes atropalpus.* When the genome of the autogenous strain was progressively replaced by repeated crossing to the anautogenous forms, a stepwise decrease in autogenous fecundity was noted. However, these intermediate levels of fecundity were rarely found in nature. For most *A. atropalpus* populations, which contained autogenous females, egg production was uniformly high, often equal to the levels obtained with blood-fed, anautogenous females. In contrast, blood-fed anautogenous *A. taeniorhynchus* females can produce much larger initial egg batches than non-bloodfed autogenous females. Moreover, among wild populations the mean autogenous fecundity varies over a wide spectrum (Table I). Results of crossing experiments between the Cayman and Rutgers strains indicate that geographical variation in the fecundity of autogenous *A. taeniorhynchus* is regulated by a genetic system which is rather similar to the one found in *A. atropalpus* (Table II). F_1 hybrids had intermediate levels of egg production, whereas the fecundity of the progeny from backcrosses to the Rutgers strain was significantly lower than the levels found in either the parental Cayman females or the F_1 hybrids.

Genotypes for high fecundity are most prevalent in populations where the majority of the females have the genetic potential for autogeny (Table I). We have conducted some preliminary selection experiments on the Rutgers strain and have increased the rate of autogeny up to the levels normally found in the Flamingo population. But the mean fecundity of this selected line of the Rutgers strain has remained unchanged. Thus there are apparently two distinct yet coadapted genetic systems involved in the production of autogenous eggs.

Unavailability of suitable hosts would surely confer a selective advantage on individuals with autogenous phenotypes. Approximately 80% of the total power aspirator collections from Flamingo was composed of non-engorged females that were both old enough and at the proper developmental stage to actively seek blood sources. Thus the primary factors restricting blood-feeding in the Flamingo population would seem to be part of the mosquito's external environment rather than some condition or mechanism operating within the female mosquito. Clearly, the Big Pine Key population was more successful at obtaining blood meals (Table IV) and the frequency and fecundity of autogenous females were significantly lower than the levels found at Flamingo. Our findings do support the hypothesis that autogenous females are abundant in some populations of *A. taeniorhynchus* because suitable hosts are either unavailable or available only on a very limited basis.

Dispersal flights and migration occasionally transport *A. taeniorhynchus* many miles inland (Provost, 1952, 1957; Bidlingmayer and Schoof, 1957). The blood-feeding characteristics of these mosquitoes can be quite different from those that remain relatively close to the coastal habitat (Edman, 1971). Migrations in salt-marsh mosquitoes have no equivalent return flight. Therefore most of these inland mosquitoes probably do not return to the salt marshes or mangrove swamps. The comparatively short life span of the adult further decreases the likelihood of a successful return flight. Since adequate inland egg laying and aquatic habitats are seldom available, most of the *Aedes taeniorhynchus* occurring several miles from the coast probably do not contribute progeny to the next generation. Thus an evaluation of the factors that select for autogeny in *A. taeniorhynchus* should be based on data derived from collections made near the coastal habitat where mosquitoes are most likely to find proper oviposition and developmental sites for their progeny.

Numerous factors can produce conditions where potential hosts become unavailable. Although adverse climatic conditions severely limit feeding in some species, these factors do not appear to be inhibiting blood-feeding in *A. taeniorhynchus* populations. In fact, many of the highly autogenous populations encounter milder climates than do the anautogenous populations. Historically, the mangrove swamps of the subtropical region tend to be more mammal depauperate than the grassy salt marshes of the temperate zone. Still several species of vertebrates, particularly birds, occur in and near the mangrove and most of them, even some that are abundant, are seldom fed upon by salt-marsh mosquitoes. Obviously, some hosts are not accessible for reasons unrelated to their densities. Certain vertebrates show highly effective anti-mosquito behavior (Edman and Kale, 1971; Webber and Edman, 1972). Such defenses often become more pronounced when attacks from mosquitoes become more intensified (Edman, Webber and Kale, 1972b). During the spring, summer and fall the mangrove swamps in the vicinity of Flamingo, Florida often produce hordes of blood-thirsty mosquitoes which would definitely evoke maximum levels of anti-mosquito defensive behavior in most vertebrates, including man. Besides active defenses, other types of host behavior can also make a potential blood source unavailable. For an animal to be an acceptable host, it must be available when the mosquito normally seeks a blood meal. *Aedes taeniorhynchus* normally seeks a blood meal only during the crepuscular and nocturnal period. The potential availability of some vertebrates is more apparent than real because during the blood seeking period these animals are in microhabitats which are inaccessible to the mosquitoes.

The mosquitoes at Flamingo utilized a wider range of hosts than did those on Big Pine Key. Yet, one group, the ruminants, although the dominant host at Big Pine Key, was not a source of blood for the Flamingo mosquitoes. Big Pine Key contains about 6,000 acres and there are approximately 275 Key deer (*Odocoileus virginianus clavium*) on the island. Unlike the Key deer, the range of the mainland race of the white tailed deer (*O. v. virginianus*) seldom includes the mangrove swamps in the Flamingo and Cape Sable area. Since more than 75% of all mosquito blood meals on Big Pine Key came from the deer, the higher engorgement rates for this population when compared to the Flamingo population can be readily attributed to differences in the availability of this single host species. The percent avian blood-feedings in the Big Pine Key population was double the rate of Flamingo females. Nevertheless, only 21% of the total feedings were from birds. Thus, the contribution of avian hosts to the overall availability of suitable blood-sources was minor when compared to that made by deer (Table VI). Rabbits appeared to represent the most readily available host for *A. taeniorhynchus* at Flamingo. Still, they could not compensate for the absence of deer in view of their much smaller size and the limited area of suitable rabbit habitat at Flamingo. Marsh rabbits were particularly common in the grassy areas associated with roadways and campgrounds near our collection sites at Flamingo but elsewhere they seemed to be far less abundant.

Corbet (1967) found obligate and facultative autogeny in *Aedes nigripes* and *A. impiger*. Females displaying obligate autogeny begin developing their eggs at emergence and are ready to lay them within 10 days. In contrast, females exhibiting facultative autogeny have their ovaries in a state of suspended development for at least 10 days before they start to mature eggs autogenously. A facultative type of autogeny has not been found in *A. taeniorhynchus*. However, the methods used in the present study would not detect facultative autogeny. Laboratory-reared, anautogenous mosquitoes might, under field conditions, be stimulated by chemicals such as phytoecdysones to produce eggs in the absence of a blood meal (Spielman, 1971; Spielman, Gwadz, and Anderson, 1971). But again there is no evidence that shows this actually occurring in any wild mosquito population. Despite the obvious limitations of our methods for detecting autogeny in *A. taeniorhynchus* populations, we were still able to demonstrate genetic differences among populations. And in southern Florida these differences are associated with distinctive blood-feeding patterns and rates.

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SUMMARY

1. A total of 17 populations of *Aedes taeniorhynchus* were examined for the occurrence of autogeny. Every population contained some autogenous as well as

some anautogenous females. In general, populations from the tropics and subtropics had higher rates of autogeny than did temperate zone populations. A major exception to this pattern was a Panama strain where less than 1% of the females were autogenous.

2. Autogenous and anautogenous females occurred in the same reproductive populations. The progeny derived from individual, wild-caught females often contained both reproductive types.

3. The mean fecundity was often less than 30 eggs per autogenous female in those populations where the occurrence of autogeny was rare or infrequent. In contrast, significantly higher levels of fecundity were associated with all populations that contained mostly autogenous females. Geographical variation in autogenous fecundity appears to be regulated by polygenic factors.

4. We compared the blood-feeding characteristics and requirements of two mosquito populations in southern Florida. At Flamingo, Florida, only 8.1% of the females collected with power aspirators were engorged, whereas at Big Pine Key, Florida the engorgement rate was 20.9%. Approximately 80% of all females from the Flamingo collections were at a stage where they could be considered "blood-thirsty" mosquitoes. In contrast, only about 60% of the Big Pine Key females were in this category. Both the frequency and the fecundity of autogenous females were significantly greater at Flamingo.

5. The higher engorgement rate for the Big Pine Key population when compared to the Flamingo population can be readily attributed to the differences in the availability of a single host species, the Key deer.

6. Our findings support the hypothesis that autogenous females are abundant in some populations of *A. taeniorhynchus* because hosts are either unavailable or available on a very limited basis.

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UPTAKE OF NATURALLY OCCURRING PRIMARY AMINES BY MARINE ANNELIDS

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Rapid, carrier-mediated influx of small organic molecules across the body wall of marine invertebrates has been studied by a number of investigators. Recent literature is reviewed by Stephens (1972) who interprets available evidence as supporting a net influx which is large enough in many instances to represent a significant supplement to the nutrition of the animals concerned. This interpretation has been questioned by Johannes, Coward and Webb (1969). These investigators studied exchanges of amino acids between the flatworm, *Bdelloura,* and the environment and found a net efflux. Stephens and Schinske (1961) studied net exchanges of amino acids in a wide range of invertebrate material and reported a net influx. These results can be reconciled by noting the very different ambient concentrations used by the two sets of investigators and the relative insensitivity of efflux to changes in ambient concentration (as argued in Stephens, 1972). However, this analysis is somewhat indirect since it is based on separate measurements of influx and efflux using labelled substrates. The present contribution provides direct evidence for a rapid net influx of naturally occurring organic material and amino acids in two genera of the annelid infauna.

A fundamental gap in the literature concerning transepidermal uptake is the failure to establish any convincing link between this process and a quantitatively tenable source of available organic compounds in the environment. Speaking more generally, transepidermal uptake needs to be integrated into the general trophic structure of marine communities in order to aspire to a status other than that of an interesting curiosity of unknown significance. The present contribution also reports stimulation of production of available organic compounds in sediments as a result of the presence and activity of the annelid infauna. This represents the required linkage between uptake and production and is an initial step in the desired integration of the process into the overall trophic structure of the community.

This work was greatly faciliated by the introduction of a new reagent, fluorescamine, which allows rapid and sensitive measurement of primary amines in aqueous solutions. Fluorescamine reacts with primary amines, including amino acids and peptides, to form highly fluorescent derivatives (Udenfriend, Stein, Böhlen, Dairman, Leimgruber and Weigele, 1972; Weigele, DeBernardo, Tengi and Leimgruber, 1972). North (1975) describes the use of fluorescamine for measurement of naturally occurring primary amines in sea water. The simplicity of the procedure facilitates data collection, and the sensitivity of the reagent is sufficient to permit investigation of naturally occurring primary amines at concentrations as low as 2×10^{-7} moles per liter (expressed as equivalent glycine concentration).

Materials and Methods

Specimens of *Capitella capitata* and *Nereis diversicolor* were collected at Kysing Fjord, an estuary on the east coast of Jutland. The locality is described in detail in Muus (1967). Water temperatures decreased from 16° to 7° C during the period of study. Animals were maintained either in an aquarium room at 15° C or a refrigerator at 6° C. Specimens for use in studies of uptake of primary amines were held in small vessels containing sea water or in aquaria containing sediment collected from Kysing Fjord.

Sediment cores were obtained from Kysing Fjord and from other areas for comparison. A clear plastic cylinder, 25 mm internal diameter and 15 to 20 cm in length, was forced into the sediment, corked and carefully removed to obtain samples for study with a minimum of disturbance of existing stratification. Interstitial water from sediment and sediment cores was expressed under 2 to 3 atmospheres of N_2 using a pressure filtration apparatus and Millipore filters of 0.45 micron pore size. This procedure expressed 60 to 70 percent of the water contained in the sediments studied.

Amino acids and naturally occurring primary amines were measured as follows. A sample of 0.5 ml was buffered to pH 9.25 with 0.5 ml of 0.5 N borate buffer. 0.5 ml of 200 mg fluorescamine per liter of acetone was added to the buffered sample mixing vigorously. The resulting precipitate was redissolved by adding 1.5 ml glass distilled water. Fluorescence was measured at 480 nm using an excitation wavelength of 390 nm. Glassware was acid washed and rinsed with glass distilled water. Fluorescamine positive material is present in Millipore filters; this was eliminated by soaking in dilute acid and in glass distilled water. High concentrations of sulfide ion interfere with the analysis. Selected samples from sediment core interstitial water were analyzed with and without addition of $CdCl_2$ to precipitate sulfide to verify that natural levels of sulfide did not interfere. Sea water solutions of amino acids were prepared using natural sea water passed through a Millipore filter. Natural primary amines were less than 5×10^{-7} moles per liter expressed as glycine equivalents.

Redox potentials were measured using a polished platinum wire electrode. The electrode was cleaned before each series of measurements and standardized using quinhydrone at pH 6.5. Water content of sediment was determined by weighing after drying at 105° C. Organic content was determined after ignition at 470° C. Free amino acids in samples of interstitial water were desalted and chromatographed using the TLC procedure described by Clark (1968). The samples were lyophilized and chromatographed in California.

Results

Concentration of primary amines in interstitial water

Concentration is expressed as glycine equivalents. As noted by North (1975) specific fluoresence of fluorescamine derivatives of amino acids differs. Using the procedure described above, I investigated the fluorescence of glycine, serine, glutamate and aspartate solutions in natural sea water. Fluorescence of the fluorescamine derivatives was found to be 1 : 0.87 : 0.67 : 0.47 for these amino acids at a pH of 9.25. Other primary amines (*e.g.*, peptides) have a rather lower fluorescence at this pH and ammonia does not give any fluorescence. North reports that the specific fluorescence of lysine, arginine and ornithine is slightly higher than that of glycine. However, these amino acids are not major constituents of primary amines in interstitial water. Thus the use of glycine equivalents represents a conservative estimate of molar concentrations of naturally occurring primary amines.

Primary amines were measured in a total of 32 samples of interstitial water from the top three centimeters of sediment. Concentrations ranged from a minimum of 29 to a maximum of 103 micromoles per liter expressed as glycine equivalents. The average value was 50 micromoles per liter. Deeper levels of the sediment cores exhibited lower concentrations of primary amines. Thus the concentrations measured at depths between 6 and 9 centimeters ($n = 8$) ranged from 16 to 40 micromolar with an average of 23. Duplicate measurements on the same extract were excellent, normally agreeing within 2 percent as did duplicates obtained by extracting split cores. However, multiple sampling over an area of a square meter indicated a patchy distribution with individual determinations of primary amines from different cores differing by as much as 50 percent. There was no obvious macroscopic correlate of this patchiness.

Because of the variation in level of primary amines in the cores examined, values for surface samples overlap with values at deeper levels, as noted above. Table I expresses data from measurements of primary amines in succssive layers of natural cores collected from Kysing Fjord. The table gives the ratio of primary amines in the 3 to 6 cm layer and the 6 to 9 cm layer to primary amines in the surface 3 cm. The decrease in primary amine concentration with depth is a consistent feature of these measurements.

Redox potentials of each core were measured at 5 mm intervals from the sur-

TABLE I

Ratios of the concentration of primary amines in interstitial water of sediment cores at the indicated depth to concentration of primary amines in the interstitial water of the surface 3 cm of the cores.

	Depth (cm)	Primary amines (micromols/1)	Ratio of amine concentrations	n
Natural cores	0–3	51.0 ± 24.8		11
	3–6	27.4 ± 14.9	0.57 ± 0.18	11
	6–9	23.1 ± 9.8	0.50 ± 0.14	8
Sieved cores	0–3	57.6 ± 28.8		6
	3–6	34.8 ± 15.5	0.61 ± 0.11	6
	6–9	26.1 ± 9.3	0.50 ± 0.12	6
Cores plus	0–3	43.9 ± 26.0		6
N. diversicolor	3–6	38.6 ± 24.2	0.90 ± 0.17	6
	6–9	39.9 ± 17.9	0.93 ± 0.37	6
Irrigated cores	0–3	55.4 ± 18.6		6
	3–6	51.6 ± 14.2	0.96 ± 0.18	6
	6–9	55.0 ± 21.1	1.00 ± 0.24	6

face. All cores examined were quite reduced, achiving an E_h of 0 mv from 3 to 12 mm below the surface and an E_h of −140 to −250 mv at a depth of 3 cm.

Net influx of naturally occurring amines and of amino acids

Single individuals of *N. diversicolor* and groups of 10 to 15 *C. capitata* were exposed to samples of interstitial water or to solutions of glycine, serine, glutamate and aspartate. Specimens of *Nereis* ranged in weight from 71 to 237 mg and the groups of *Capitella* were selected to weigh approximately 100 mg collectively.

Primary amines were determined initially and at suitable intervals after immersing the worm or worms in a sample. The general result was an exponential decrease in concentration of primary amines with time. Figure 1 is a semilog plot of the concentration of primary amines in interstitial water from a surface (0 to 3 cm) sample taken from Kysing Fjord and exposed to a group of *C. capitata.* Weight of worms was 111 mg and the volume of the water sample was 10 ml. In this particular case, the worms had been selected and exposed previously to six aliquots of the same interstitial water sample at approximately six-hour intervals over the preceding two days. Sufficient streptomycin sulfate and penicillin was added to give concentrations of 100 mg/liter and 10^6 IU/liter respectively. The rate of influx expressed as micromoles of glycine equivalents per gram per hour can be estimated from the slope of the semilog plot and is approximately 0.81 from a concentration of 50 micromoles (glycine equivalent). This is not significantly different from the rate of influx observed when naive worms are exposed for the first time to the same interstitial water sample without antibiotics (see Table II).

As indicated in Table II naturally occurring amines were also available to *Nereis* at a comparable rate. However, the data for the two species differ. The exponential decline in concentration of primary amines can be followed down to

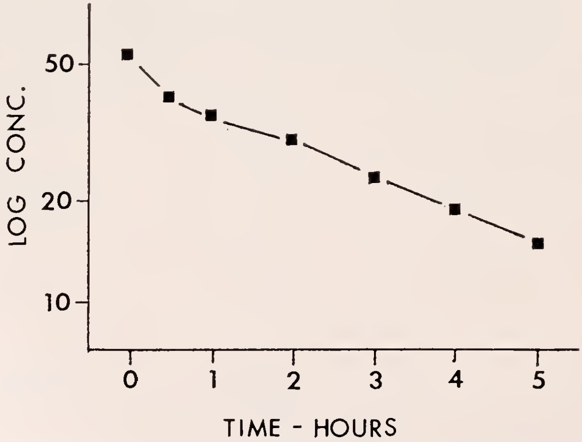

FIGURE 1. Disappearance of naturally occurring primary amines in the presence of *Capitella capitata.* Initial concentration is 54 micromoles per liter (glycine equivalents); final concentration is 15 micromoles per liter. See text for details.

TABLE II

Net influx of primary amines from interstitial water and net influx of amino acids. Rates are expressed as micromoles per hour per gram worm from an ambient concentration of 50 micromoles per liter.

Capitella capitata		*Nereis diversicolor*	
amines*	0.89	amines*	0.75
amines**	0.81	glycine	0.84
glycine	0.75	serine	0.99
serine	0.85	glutamate	0.05
glutamate	0.82	aspartate	0.03
aspartate	0.85		

* Expressed as micromoles glycine.

** Incubated for 48 hours with streptomycin and penicillin, seventh exposure to amines.

concentrations of 2 to 3 micromoles per liter in the case of *Capitella.* At this point influx ceases and concentrations may increase to 4 to 5 micromoles over the course of several hours. In contrast, external concentration of amines decreases exponentially in the presence of *Nereis* until a value of 30 to 40 percent of the initial concentration has been reached. The rate of disappearance declines slowly. This is to some extent independent of initial concentration since the same result is obtained if the naturally occurring amines are diluted to present an initial concentration of 10 micromoles.

Net influx of known acids was examined using *Nereis* and *Capitella.* Initial concentrations were 50 micromoles per liter (10 in some experiments). Table II gives rates of net influx from a concentration of 50 micromoles per liter. *Capitella* obtained glutamate and aspartate from solution at rates comparable to those observed for glycine and serine. The values of glutamate and aspartate uptake are on an order of magnitude lower for *Nereis.* It is not clear that *Nereis* could take up these compounds at all. The data show a consistent downward trend but the experiments were relatively long (8 hours) and no antibiotics were used. Thus *Nereis* makes little or no use of the free dicarboxylic amino acids present in the interstitial water while *Capitella* can apparently do so. *Nereis* reduces the ambient concentration of glycine or serine to minimum levels of 1 to 3 micromoles per liter; *Capitella* does so for all of the four amino acids employed.

Free amino acids in interstitial water samples

Fifteen amino acids were identified from two samples of interstitial water which were desalted, chromatographed and eluted for quantitative estimates. The order of concentration in the two samples was respectively ala, glu, gly, ser, val, asp for the first and ala, glu, asp, gly, ser, val for the second. Amino acids accounted for virtually all of the ninhydrin positive material in the desalted samples. The data do not permit conclusions about the extent to which fluorescamine and ninhydrin determinations of primary amines and of amino acids agree. An aliquot of one of these samples which had been exposed to a group of *Capitella* for five hours was also chromatographed. Free amino acids were markedly lower in concentration. The amino acids ala, gly, glu, and ser were present in that order by visual estimate plus small amounts of lys, arg, and his.

Artificial sediment cores

Eighteen artificial sediment cores were prepared by filling clear plastic tubes, 25 mm in internal diameter, to a depth of about 15 cm with sediment freshly collected at Kysing Fjord. The sediment was passed through a 1 mm mesh seive to remove macrofauna and large particles. The sediment contained 0.86% organic matter by weight as determined by ignition at 470° C. After preparation, the artificial cores were kept submerged in aerated sea water in an aquarium at 15° C. The bottom of each tube was firmly corked with a rubber stopper; the top was exposed to the aerated sea water.

All of the artificial cores became oxidized at the surface and assumed a redox profile comparable in detail to that described earlier for natural cores in this study. The first E_h measurements were taken four days after constructing the cores.

Six cores were left undisturbed as described. A single specimen of *N. diversicolor* (weight range from 96 to 243 mg) was added to each of six cores immediately after preparation. A piece of 1 mm mesh nylon screen was tied over the mouth of each tube to retain the animals. The remaining six cores were exposed to a subsurface flow of aerated sea water at a rate of 12 to 15 ml per hour. The sea water was siphoned from an aerated tank and entered the core through a Pasteur pipet. Flow rate was controlled by a tubing clamp. The tip of the pipet was inserted 6 to 7 cm below the surface of the core. The pipet was surrounded by a sleeve of nylon screen extending to the surfac of the core to minimize mechanical disturbance of the sediment as a result of the water flow.

The cores containing *Nereis* and those artificially irrigated with aerated sea water developed much the same redox profiles as the natural cores and the undisturbed artificial cores with minor differences to be described. Figure 2 shows the redox profile of a core containing a specimen of *Nereis*. In this case, as in all of the worm-containing cores, a layer of bright yellow sediment formed immediately around the burrow constructed by the worm. The solid line shows redox potential measurements away from the immediate area of the burrow; the dotted line shows measurements obtained when the platinum electrode was thrust down the burrow itself. Figure 3 shows a redox profile obtained from a core irrigated with aerated sea water. The two open squares are measurements of E_h from the same core taken with a freshly cleaned platinum electrode immediately after exposing bright yellow sediment to the air at the depths indicated.

The redox profiles of natural cores and those assumed by artificial cores imply transition from aerobic to anaerobic conditions within the top 3 cm of the sediment (Fenchel, 1971). The platinum electrode used for measurement of redox potentials is easily poisoned. However, measurements along worm burrows or in the freshly exposed, bright yellow regions of subsurface sediment in irrigated cores though higher than the surrounding sediment were still strongly negative. This suggests that oxygen introduced into deeper layers of the sediment, artificially or as a result of the activity of the worms, is transient and results in oxidation of sulfide in the immediate vicinity. In effect, an additional aerobic-anerobic transition area is produced.

Table I presents the concentration of primary amines at various depths and lists ratios of the primary amines found at depths of 3 to 6 cm and 6 to 9 cm to primary amines in the surface 3 cm for the three types of artificial cores. The

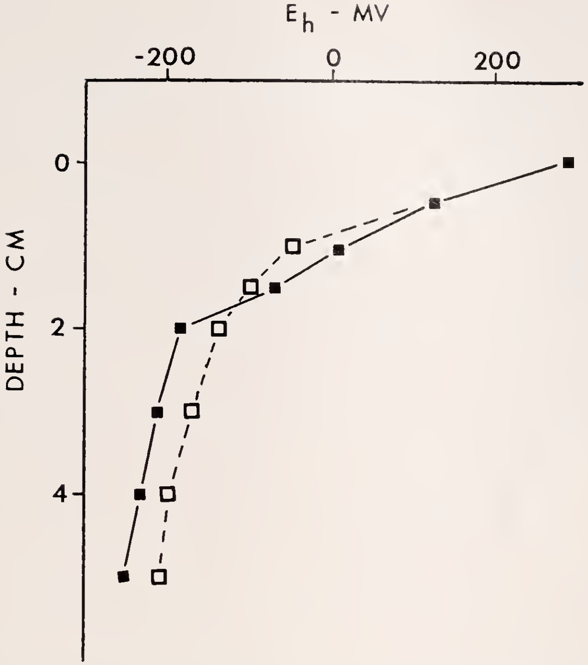

FIGURE 2. Redox profile of an artificial sediment core containing a specimen of *Nereis diversicolor*. The solid line connects measurements of redox potential of the sediment. The dotted line connects measurements of redox potential along the burrow of the worm.

content of primary amines did not decrease with depth in cores containing worms or in cores receiving a flow of aerated sea water. The average content of primary amines in the surface 3 cm of the different types of artificial cores did not differ significantly. Undisturbed and "aerated" cores averaged 57.6 and 55.4 micromoles per liter respectively. Cores containing worms averaged 43.9 micromoles per liter (range 23.3 to 92.5). Thus the increased ratios in Table I result from an increase in primary amines in the deeper layers of the artificial cores containing worms or receiving a subsurface supply of aerated sea water.

DISCUSSION

There is a net flux of naturally occurring primary amines into both *Capitella capitata* and *Nereis diversicolor*. The flux is almost certainly transepidermal though the present work does not directly confirm this deduction. If intake across the gut surface were to be postulated, the measured rates of disappearance of primary amines would imply passage of at least fifteen to twenty times the volume of the worms through their digestive tract each hour. Earlier demonstration for other annelids of rapid transepidermal influx using ^{14}C-labelled compounds at rates unimpaired by ligation of mouth and anus (*e.g.*, Stephens, 1963) as well as examination of influx in isolated epidermal preparations (*e.g.*, Chien, Stephens and Healy, 1972) support this interpretation of the present observations.

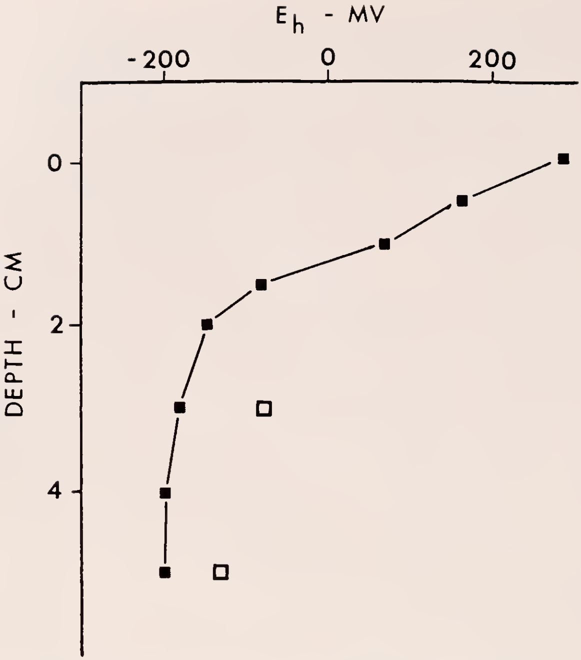

FIGURE 3. Redox profile of an artificial sediment core irrigated with aerated sea water. The solid line connects measurements of redox potential of the sediment. Open squares are measurements of the redox potential of freshly exposed, bright yellow sediment at the indicated depths.

The data strongly suggest that the worms are the agents for disappearance of the primary amines rather than contaminating microflora. This is supported by the fact that antibiotics failed to modify rates of net flux (Figure 1 and Table II). It is also supported by the fact that glutamate and aspartate were removed rapidly from solution by *Capitella* and slowly or not at all by *Nereis*. No antibiotics were present in the solutions in these experiments. It is unlikely that contaminant microorganisms were present and active in the first case and absent or inactive in the second, since the two organisms were collected from the same locality and the same sediment samples and were treated similarly.

The entry of naturally occurring primary amines occurs at rates which are of the same magnitude as the total requirement of the animals for reduced carbon. As noted above, use of equivalent glycine concentration units is a conservative estimate of abundance of these compounds. Assuming that *Capitella* is taking in the six most abundant amino acids found in interstitial water (average molecular weight, 111) the net influx reported in Table II represents 90 to 99 micrograms per gm worm per hour. Inclusion of a correction for differences in specific fluorescence would increase this figure. The same calculation for *Nereis* produces a lower figure since glutamate and aspartate are not utilized (average molecular weight, 96; hourly influx, 72 micrograms per gm). The conversion factor for a mixture of amino acids to oxygen consumption is one microgram equivalent to one microliter. Oxygen consumption data for marine infaunal annelids tabulated in standard handbooks covers a range of about 8 to 135 microliters O_2 per gm per hour.

This discussion is subject to the caveat that primary amines are not necessarily free amino acids. Previous work with annelids and other groups of soft-bodied invertebrates has shown that amino acids entering via the transepidermal route enter all aspects of the metabolism of the organism involved (reviewed in Stephens, 1972). It is possible that the naturally occurring primary amines include compounds that can enter the worms but cannot participate in their metabolism. The data from chromatography are of some help but are not conclusive in resolving this issue. Thus, little or no ninhydrin-positive material remained at the origin in chromatograms of desalted samples of interstitial water. All ninhydrin-positive spots were identifiable as amino acids. This suggests free amino acids make up the greatest part of the naturally occurring primary amines. This suggestion is also supported by the agreement between the present report of levels of primary amines and previous reports of free amino acid concentrations from interstitial water samples (Stephens, 1963; Stephens, 1972). Ammonia does not react with fluorescamine. Thus the present interpretation of primary amines as amino acids is not likely to be an overestimate of rates of entry of reduced carbon. However, it is possible that some amines may be present which are not available to the worms metabolically.

The potential contribution of this process to the nutrition of *Capitella* is of particular interest. There is mounting evidence for the view that the only significant primary decomposers of plant material in the sea are microbial (reviewed in Fenchel, 1972). Thus "detritus-feeding" metazoans are dependent on microbial activity; a number of such animals have been shown to utilize the microbial flora as a food source rather than detritus itself. Conversely, there is no convincing demonstration of the ability of a "detritus-feeder" to digest detritus. However, there are cases, one of which is *Capitella*, of animals where careful examination has failed to produce clear evidence of ability to utilize either detritus or the microbial flora. In aquaria experiments *Capitella* selectively feeds on decaying fragments of *Ulva* in the reduced sediment. However, direct observation under the microscope of stained fragments shows that many bacteria pass undigested (or they are added from the gut flora). The fragments themselves are also relatively little digested. In agreement, the total organic matter as well as the content of protein of the fragments are only slightly lower than values obtained with control fragments picked from the sediment (personal communication, J. Hylleberg, University of Aarhus). Though the possibility that *Capitella* is obtaining some sustenance from ingestion of sediment is not totally excluded, it is attractive to visualize the intake of dissolved material as a large component of its nutritional strategy. These comments do not apply to *Nereis*. Worms in this genus have been shown to digest microbial flora, diatoms and algal tissue, and the worms are opportunist carnivores and occasional filter feeders. However, the present work indicates that *N. diversicolor* is also capable of obtaining supplementary nutrition from dissolved compounds in the interstitial water.

Generation of primary amines in cores containing *Nereis* or irrigated with sea water is of great interest. The following interpretation of this observation is suggested. The redox potentials of natural cores and undisturbed artificial cores indicate that primary amines are most concentrated in the region which includes the transition zone from aerobic to anaerobic conditions. The presence of a worm or artificial irrigation has the effect of increasing the surface area of this transition zone by folding and extending this zone into the deeper layers of the sediment. Both *Capitella* and *Nereis* are known to irrigate their burrows (Lindroth, 1941). The burrowing activity of the infauna and their behavior in irrigating their burrows serves both to supply them with oxygen for their metabolism and to generate primary amines which are then available as a nutritional source. Thus the ability to obtain and utilize primary amines from a solution is coupled to the generation of these compounds.

The observations reported here do not permit identification of the source of the additional primary amines. However, the presence of detritus-feeding invertebrates has been shown to promote microbial activity in marine sediments. This is true whether one measures oxygen consumption of the microflora (Hargrave, 1970) or counts the number of bacteria per unit surface of detritus particles (Fenchel, 1972). It is likely that the present observation of an increase in primary amines is also related to stimulation of microbial activity. Jørgensen and Fenchel (1974) have shown that the activity of bacteria which reduce sulfate to sulfide and reoxidize the resulting sulfide dominates the metabolism of reduced marine sediments. Furthermore, this activity is most intense in the transition layer between aerobic and anerobic conditions. Thus it is attractive to speculate that the increase in area of this transition zone resulting from the irrigation by the worms, the intense microbial activity, and the increase in primary amines are related phenomena.

This work was carried out during tenure as a Senior Science Fellow of NATO in the autumn of 1974. I wish to express my sincere thanks to Professor Tom Fenchel for use of laboratory facilities at the University of Aarhus and for helpful discussions, to Dr. Jørgen Hylleberg for his interest and help and for permission to allude to as yet unpublished observations of feeding in *Capitella*, and to Dr. Bo Barker Jørgensen for help and advice and for obtaining sediment cores from the Limfjord. I also wish to acknowledge with thanks the contribution of Patricia Wheeler who chromatographed the amino acids in interstital water samples.

SUMMARY

1. The interstitial water of inshore sediments on the Danish coast contains rather high concentrations of primary amines. The average concentration observed was 50 micromoles per liter expressed as equivalent glycine concentration.

2. The concentration of primary amines decreased in deeper layers of the sediment.

3. Naturally occurring primary amines from interstitial water are taken up rapidly by *Nereis diversicolor* and *Capitella capitata*. The rate of net influx is of the same order of magnitude as the total requirement for reduced carbon provided primary amines are interpreted as principally consisting of free amino acids.

4. Net influx of known amino acids was also examined. *Capitella* takes up glycine, serine, glutamate and aspartate at rates comparable to those observed for naturally occurring amines in the interstitial water. *Nereis* takes up glycine

and serine at comparable rates but obtains glutamate and aspartate from solution very slowly if at all.

5. Subsurface irrigation of sediment samples with aerated sea water increases the concentration of primary amines in the interstital water of deeper sediment layers. The same effect results when *Nereis* constructs and irrigates a burrow.

6. These observations support the hypothesis that a large net transepidermal influx of small organic compounds occurs in members of the annelid infauna. Furthermore, this ability to obtain and utilize primary amines from solution is associated with behavior which results in generating additonal primary amines.

7. It is suggested that the construction of burrows and their irrigation by the infauna serves to increase the surface area of the transition zone between aerobic and anaerobic conditions by extending it into deeper sediment layers. The observed increase in primary amines may be related to the intense microbial activity which characterizes this transition zone.

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CAUSES OF DAILY RHYTHMS IN PHOTOSYNTHETIC RATES OF PHYTOPLANKTON. II. PHOSPHATE CONTROL OF EXPRESSION IN TUNDRA PONDS

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This paper continues to explore the cause and significance of daily photosynthetic rhythms in the phytoplankton. In earlier communications a model basis for the existence of an endogenous component in photosynthetic rhythms was described (Stross, Chisholm and Downing, 1973). The phytoplankton from the same lake (Lake George, New York) as elsewhere (Malone, 1971) has been shown to contain sub-components that function maximally at different times of day (Stross and Pemrick, 1974). In previous analyses rhythms were identified that were based on Michaelis coefficients resulting from the addition of various concentrations of phosphate. A photosynthetic potential was identified which measures the maximum stimulation resulting from the addition of a single, presumably rate limiting, nutrient. Reported here is an examination of the response of Lake George phytoplankton to nutrient enrichment at various times throughout the year. Secondly, the use of phosphate enrichment to identify daily maxima in photosynthetic rates was investigated in the phytoplankton from arctic pools.

A general model of the cell cycle includes a growth rate cycle only loosely coupled to cell division (Mitchison, 1974). In synchronous cultures of algae, cells divide at a predictable phase of the light-dark cycle, as is generally known. Nutrient limitation may permit only some of the population to divide at a scheduled time each day. Although cell division in such circumstances is a multi-day cycle, metabolic activities, as measured by phosphate uptake and photosynthetic rates, may oscillate with a daily frequency (Chisholm, 1974).

Application of a general model to an entire assemblage such as the phytoplankton has not been discouraged. Success of the so-called short term bioassay of Ryther and Guillard (1959) is proof that some species of the phytoplankton may readjust photosynthetic rate in response to nutrient enrichment within a 3 or 4 hour time span. Moreover, the coincidence of a maximum and minimum of photosynthetic capacity suggests that the most active taxa in the phytoplankton are synchronized (Stross *et al.,* 1973). An immediate feedback of the functional processes in all populations of the algae to resource availability is often postulated (Doyle and Poore, 1974; Ganf, 1974). Indeed, the environmental stimulus or driver is believed to be the cause of the rhythmic behavior of a functional process such as rates of uptake. The question, however, is to what extent does the amplitude and phase of oscillations in cellular activities such as cell division, nutrient uptake, and photosynthetic capacity of phytoplankton reflect the driving effect of external stimuli and to what extent are they predetermined by the inherent characteristics of the individual taxa in the phytoplankton? The individuality of many species in culture is summarized by Senger (1970) and Chisholm (1974).

In the arctic and near arctic where the day is all lighted and the sun may be continuously above the horizon, evidence for rhythms in the activities of algae have included some of the most precise observations on field rhythms (Müller-Haeckel, 1970a, b; 1971). Rhythms in rates of cell division and attachment to substrata have been recorded for many species of algae that live in streams. Attachment rates of diatoms are demonstrated to be under the control of an endogenous clock (Müller-Haeckel, 1971).

The arctic coastal plain in northern Alaska where the study was conducted is characterized by low relief and elaborate development of a frost-patterned earth (Brown, 1970). Ponds may develop in the centers or between the polygonal patterns that result from frost action. In either case the ponds contain typical ecological communities, and the species are similar to those in temperate latitudes with the exclusion of vertebrates. Included in the community is a diverse assemblage of algae which has been intensively described for the area near the Arctic Research Laboratory at Point Barrow, Alaska (Kalff, 1967a, b; Alexander and Coulon, 1973).

METHODS

Photosynthetic capacity was estimated as the net apparent rate of carbon fixation with minor modification of the standard technique. Samples of 100 ml were incubated for three hours on a revolving drum incubator (PECCER) at approximately 1500 ft-c and at the surface temperature of Lake George. In the arctic studies, either a revolving drum or cabinet incubator was employed at a temperature of 12° C ± 0.5 in each case. A correction of 1.43 × was applied for excretion by Lake George phytoplankton after extensive measurements (Downing, T. A., S.U.N.Y., Albany, unpublished manuscript). The arctic estimates were uncorrected.

Water from Lake George, New York was collected from 2.0 m beneath the surface immediately before the first of each time sequence of measurements. After passing through a number 25 mesh net (aperture = 64 micrometers), the water was transported and stored in 20-liter Pyrex carboys. The carboys, suspended at the surface of the lake and shielded from the direct rays of the sun, were agitated before removal of samples. Water from adjacent arctic pools (Ponds B and C USIBP Site 7) was collected from the pond surface and handled in a similar manner. The location of storage was at the Naval Arctic Research Laboratory, away from the ponds. The manner of storage was part of the experimental design.

At incubation, samples were enriched with phosphate only or with a "complete" set of nutrients routinely used for the culture of auxotrophic algae but more dilute (Stross *et al.*, 1973). Phosphate was tested at one or more concentrations ranging from 0.1 to 2.0 micromolar.

Statistical analysis consisted of a multiway analysis of variance and comparison of means (Sokal and Rohlf, 1969).

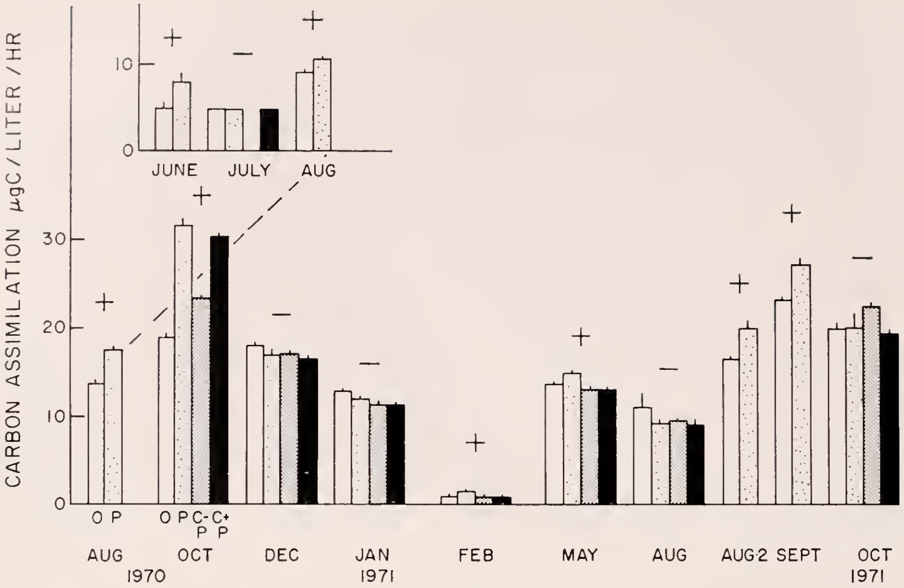

FIGURE 1. Rate of net carbon assimilation (PC) at 1500 ft-c and ambient lake temperature in response to nutrient addition. Shown are results from a sequence of tests beginning in June, 1970 and extending to October, 1971. Water was taken from Lake George, New York, station 1 and station 6 (upper left). Measurements were performed between the hours of 1000 and 1500. When phosphate stimulated ($P \leq .05$) photosynthesis, the sequence is marked with a "+". Symbols: O represents no additions; P, 0.25 or 0.5 μM PO_4 as sodium (primary) phosphate added; C-P, a complete nutrient solution added minus phosphate (see Stross *et al.* (1973) for details of the solution); C + P, complete nutrients including phosphate.

RESULTS

Continual bioassay of Lake George phytoplankton revealed that added phosphate stimulates photosynthesis as described earlier (Stross *et al.*, 1973). However, an apparent stimulation was observed on six of ten sampling dates only (Fig. 1). Hourly rates of carbon uptake ranged from 23.0 to less than 1.0 μg C/liter/hour. The addition of phosphate stimulated photosynthesis at each extreme of the range. There was, in other words, no clear association between the response to phosphate and the photosynthetic capacity of the phytoplankton.

A complete set of nutrients with or without phosphate was tested on seven dates at various times during the year. It was stimulatory on two occasions, once clearly the result of the included phosphate (Fig. 1; October, 1970) and once by some nutrient other than phosphate (October, 1971). Repetitive measurements were made on most of the sampling dates. The response was consistent however, and only the time of the expected daily maximum is shown in Figure 1.

The phytoplankton of the myriad small pools of the tundra near Barrow, Alaska is reasonably diverse in species but of extremely small numbers of cells (Kalff, 1967a, b; Alexander, Clasby and Coulon, 1972). Rates of photosynthesis at constant light and temperature ranged from 0.08 to 2.0 μg C/liter/hour in the analyses reported here which began in 1971. Alexander and Coulon (1973) reported a considerable seasonal oscillation with a maximum after ice melt (mid-June) and again in the arctic autumn (August) of approximately 12 μg C/liter/hour. The minimum rates occurred in July, and their results are within the range of the rates reported here. Alexander and Coulon (1973) also report the dominant species of algae in the phytoplankton, which is dominated by species of the Cryptophyta and to a lesser extent by species of Chrysophyta and Chlorophyta.

Daily oscillations in the photosynthetic capacity are reputed to have small amplitudes in the arctic (Doty, 1959). The experiments were designed to detect an oscillation and to determine if the reputed small amplitude is the result of

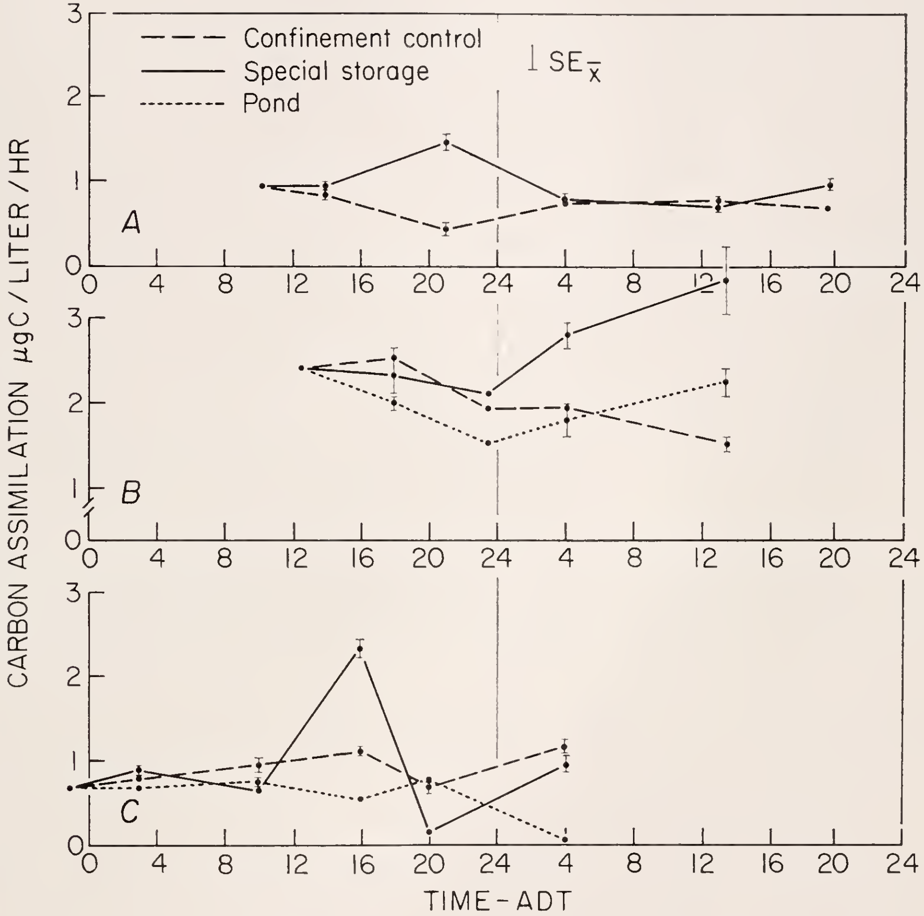

FIGURE 2. Daily time course of photosynthetic capacity (μgC/liter/hr) of arctic phytoplankton when samples removed from an arctic pond each time or from storage carboys exposed to daylight and varied temperatures (confinement control, spaced dashes) or from special storage (special, broken line). A, B and C are individual sequences begun on different days in July 1971 and started at different hours of the day. See text for details.

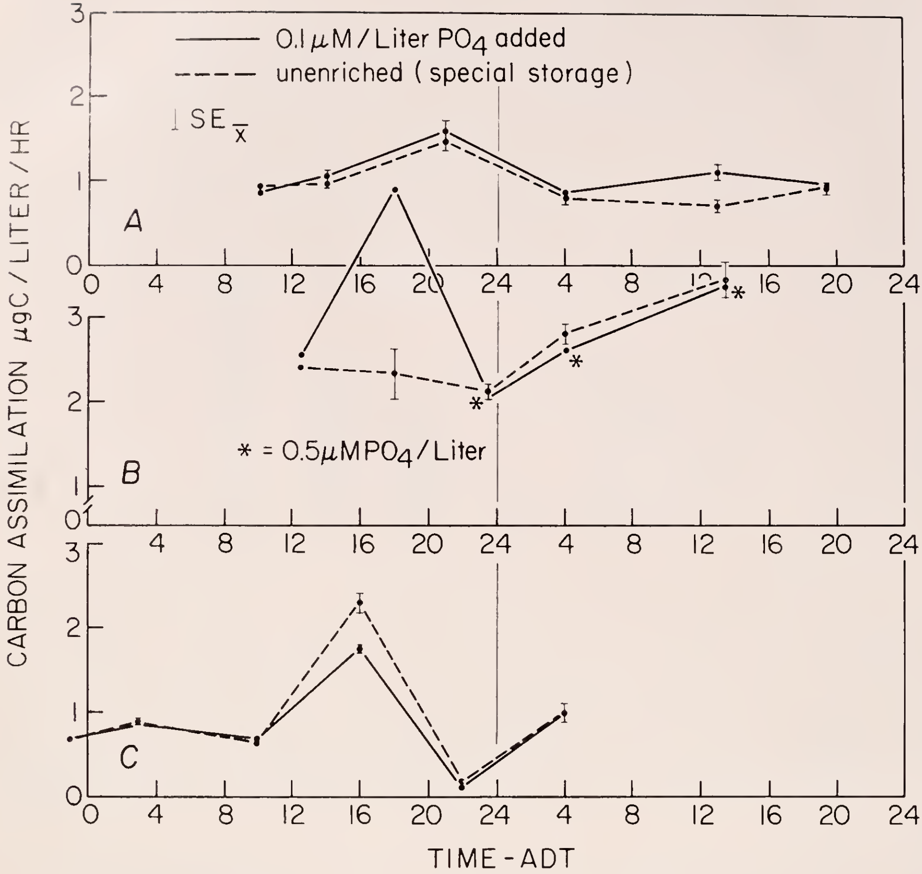

FIGURE 3. Photosynthetic capacity with and without phosphate enrichment added at the time of incubation. Unenriched controls (dashed line) are the special storage shown in Figure 2. Solid line shows a second part of the same sample when enriched with phosphate and incubated with the controls. Note that dramatic stimulation was restricted to only one of 17 measurements, that shown at 1800 in B.

extrinsic or intrinsic regulation. Two 20 liter carboys of water were removed from one of two tundra ponds at the beginning of each experiment. One carboy, the confinement control, was held outside of the laboratory usually shielded from the sun, when present. Samples from this carboy were paired with samples taken before each run directly from the pond. A second carboy was placed in a constant temperature (5° C) room at low light intensity (*ca.* 50 ft-c). The low temperature was similar to the morning minimum temperature of ponds at Barrow (71° N) where temperatures oscillate between 5° and 15° C on clear days.

Small fluctuations in the daily pattern of photosynthetic capacities were evident in samples taken directly from the pond (Fig. 2; dotted line) and suggest a small amplitude only. The confinement control also provided similarly phase indistinct, although statistically significant, fluctuations.

Large oscillations in photosynthetic rates were clearly evident in samples from the "stored" carboy. The peak in two of three runs was measured at 1600 and 2000. In a third run (Fig. 2B), storage in dim light-low temperature resulted in larger rates the following day, however. The late afternoon peak, although absent, was latent, as shown by the response to added phosphate.

Enrichment with phosphate (0.1 and 0.5 μM/liter) stimulated photosynthesis significantly on only one occasion in the three runs of July, 1971 (Fig. 3B). The single instance of nutrient-stimulated photosynthesis resulted in a distinct daily maximum in samples where a peak was expected.

It may be concluded that photosynthetic peaks in the arctic are suppressed. Special handling of the water samples allows expressions of a daily maximum, however, with amplitudes comparable to those observed at temperature latitudes. A common denominator in the successful treatment could be a charging of the algal cell with one or more nutrients. Although possibly coincidental, the special storage was insufficient in the run where placement of the carboy was delayed to mid-morning. Phosphate enrichment somehow substituted for the delayed storage. The time of the daily maximum, although not precisely determined, was in the vicinity of 1800. A "supper-hour" peak is later than is usually observed in temperate or tropical environments (Doty, 1959; Lorenzen, 1963; Malone, 1971).

A subsequent experiment was designed to test the inference that storage of the phytoplankton in a dim light-low temperature environment allows internal nutrients levels, specifically phosphate, to accumulate, thereby permitting faster rates of photosynthesis. Pairs of carboys were handled as above, one remaining exposed to the mostly indirect sun light, the second stored in constant conditions. One pair was started at 2200, a second at 0700 (ADT—Alaska Daylight Time). A fifth carboy was enriched with 0.1 μM/liter sodium (primary) phosphate and exposed to daylight with the confinement controls.

Photosynthetic capacity underwent a daily oscillation in the phytoplankton from all treatments (Fig. 4A). In the confinement controls, a peak of 0.11 μg C/liter/hour was measured at 1300 and nearly that at 1800. In the stored samples the daily maximum of 0.18 μg C/liter/hour was clearly at 1800 and apparently independent of the time the treatment began. In the "late storage" sample, the rate declined to a much lower level in the 2300 measurement (Fig. 4A).

A daily maximum was also evoked at the expected time in samples from the phosphate-enriched carboy. The rate, although less than in the two stored samples, showed the same general magnitude of increase from the previous measurement. The first measurement at 1300 was clearly suppressed below that measured in the confinement controls.

The pattern of response to phosphate enrichment although not completely consistent suggested that suppression of photosynthesis was characteristic the first day of confinement, while later measurements indicated stimulation (Fig. 4B).

A repeat experiment confirmed the major results. The daily maximum was again at or near 1800 ADT. Secondly, manipulation of the pond sample was essential to expression of the daily maximum. Thirdly, either storage in the special environment or preliminary enrichment with phosphate was capable of evoking the maximum. Fourthly, the addition of only 0.1 μM/liter phosphate was suppressive during the first day of confinement. In addition to confirming

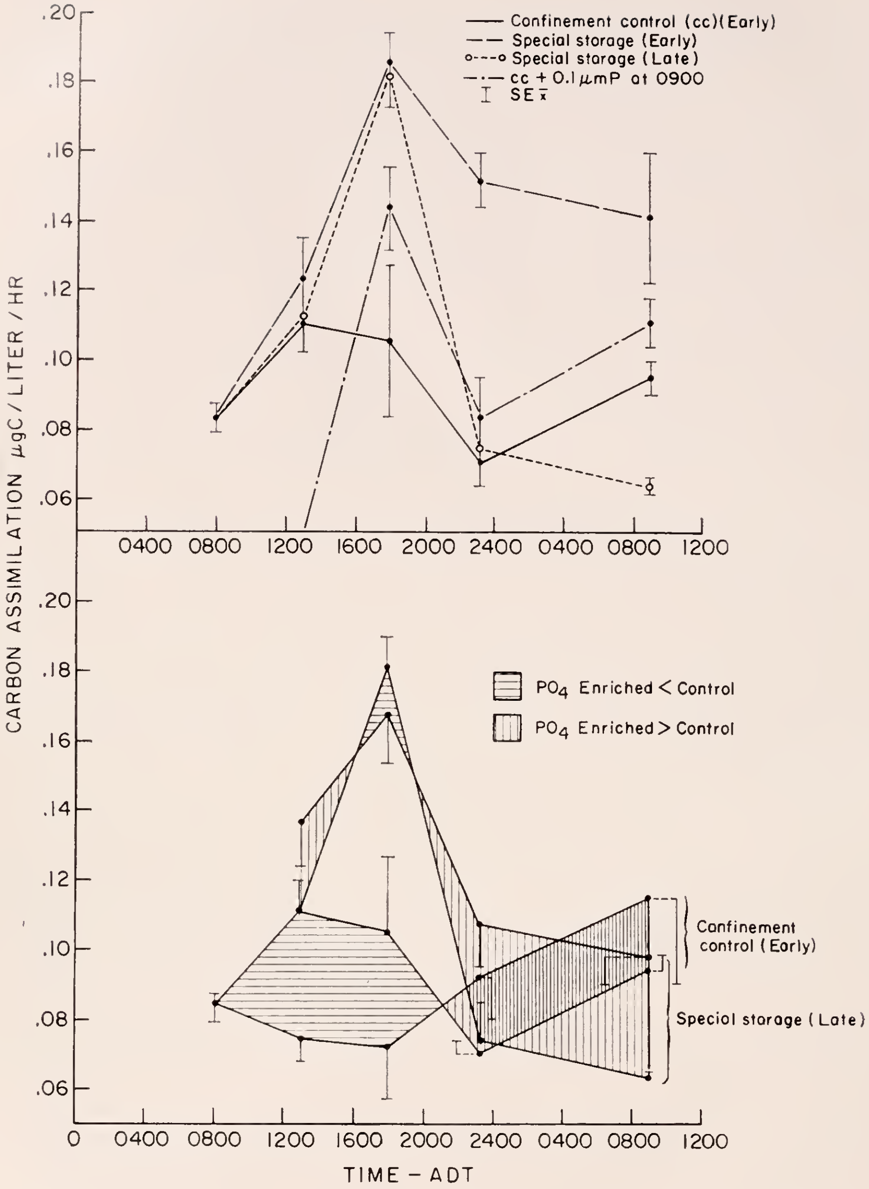

FIGURE 4. Daily time course of photosynthetic capacity in the first experiment of 1973. Shown (A) are the rates of photosynthesis in confinement control (solid line) as compared with rates following preincubation in dim light beginning at 2200 (dashed line) or 0700 ADT (dotted line). Compared also are the photosynthetic rates in samples taken from a carboy treated as a confinement control and enriched with 0.1 μM/liter sodium phosphate (dash — dot line). Compared in B are samples enriched with 0.1 μM/liter PO_4 with unenriched samples. Both confinement control and special storage treatments were chosen for comparison.

the first, the second experiment showed that although confinement and storage were made at 2100 on day zero the daily maximum took place at the expected time of 1800 on the day following.

Two general patterns of response were apparent in the three summers of observation. In 1971 and 1973 the daily maximum was late in the afternoon (supper hour). The immediate reaction to phosphate during the day was non-stimulatory and even suppressive. In 1972, photosynthesis was maximum in late morning or mid-day. Moreover, the addition of phosphate was generally stimulatory whenever samples were enriched. Both climate, which was warmer in 1972, or taxonomic peculiarities are possible explanations for the pattern differences in the time of the daily maximum and in the timing of the reaction to nutrient enrichment.

Discussion

Photosynthetic performance of the phytoplankton is similar in temperate lake and arctic pond. In each location photosynthetic capacity was observed to oscillate over the course of the day (Stross *et al.,* 1973 and above). In each location photosynthesis was stimulated within a three-hour interval following phosphate enrichment. Fertilization was effective on only some of the test dates, however. When not immediately effective with arctic phytoplankton, phosphate was still obviously stimulatory. Moreover, the restriction of a positive response to a particular time of day clearly implicates a biological rhythm. Interpretation of that rhythm is both critical and controversial.

Alternative viewpoints prevail as regards the cause of daily photosynthetic rhythms. One alternative views the phytoplankton as an externally driven system with or without a lag. Under its aegis, nutrient refractory rates of photosynthesis, as measured by the short-term bioassay (Ryther and Guillard, 1959), or decline in chlorophyll concentration as the sun rises in the sky (Yentsch and Ryther, 1957; *cf.* Ryther, Menzel and Vaccaro, 1961), can safely be viewed as direct responses to the nutrient and light environments. Morning "pulses" and afternoon "naps" imply direct reactions to a nightly increase in concentration of nutrient and photo-inhibition, respectively. Another alternative argues a certain freedom of responsiveness for the algae involved.

Extrapolation of the laboratory to the field permits a model combining endogenous rhythms with the kinetics of metabolic rates. Such a model allows metabolism to oscillate in a constant nutrient environment but with light-dark cycles. Nutrient uptake and growth rates are proportional both to concentrations in the external and internal environment and to the coefficients that relate uptake activity to substrate concentration (Dugdale, 1967; Caperon, 1968; Eppley, Rogers, McCarthy and Sournia, 1971; Fuhs, 1969; Rhee, 1973). However, the coefficients, for example, U_{max} and K_m are seen to oscillate with a daily periodicity in both saturated and nutrient limited cells of algae. Actual performance is then modified by the phase of oscillation, as demonstrated by Chisholm (1974). In other words, growth rate or rate of carbon assimilation is measuring both substrate concentration and phase of the daily cycle. O'Brien's (1972) models of growth are unrealistic because they do not specify physiological time dimensions.

An interpretation of the performance of arctic phytoplankton requires one more modification of the daily oscillation model. The "shape" of the daily oscillation may need to be something different from the sinuosidal which has been used following the suggestion of Eppley *et al.,* (1971). Nutritionally charged phytoplankton, following incubation in sub-saturation intensities of light, developed a single pulse, often with rates three times larger than values taken previous to, or following, the peak. The single pulse was thus not more than a few hours in duration, at a predictable time of day, and was preceded by a long interval with a uniform rate. Within the latter interval is a period in which a nutrient may suppress rather than stimulate photosynthesis.

Cell division cycles in arctic algae may be phased to the earth's rotation (Müller-Haeckel, 1971). The presence of a daily maximum in photosynthetic rate is not surprising. Expression of the maximum is apparently uncertain and presumed to be nutrient limited since several manpulations were associated with the evening maximum in the experiments. Phosphate is suspected to be the limiting nutrient, since the addition of phosphate at an appropriate time was sufficient to stimulate an evening peak, albeit smaller than that which resulted from more elaborate manipulations.

It is the delayed, time-specific response to phosphate enrichment that argues for an endogenous phasing of the evening maximum. Why, for example, should samples exposed to light, although rarely direct sunlight, erupt at the same time as samples stored all day at only 50 ft-c? The delay in response to enrichment was also associated with the timing of the daily maximum. Peak rates in the vicinity of 1800 (ADT), the "supper hour," were observed as a part of a pattern that included a delay between nutrient enrichment and photosynthetic response on one hand, and a likely suppression in photosynthesis immediately following enrichment on the other.

A distinct pattern prevailed in 1972 and was different from that observed in the summers of 1971 and 1973. The daily maximum was near noon and the addition of phosphate usually stimulated photosynthesis within the three-hour incubation period. This latter condition of immediate response to phosphate may be the more typical of cultures in cyclostats (Chisholm, 1974), either because of the environment or the genotype selected.

An alternative explanation seems necessary to account for the typical pattern in arctic phytoplankton. The model described above may need to include a time coupling between the photosynthetic rhythm and a nutrient-controlled internal regulator of carbon assimilation. Such a model could simulate "a nutrient limited but rarely a phosphorus deficient, cell" (Peterson, Barlow and Savage, 1974).

A consequence of the time lag between enrichment and response is the need to reevaluate the so called short-term bioassay of Ryther and Guillard (1959). Only a positive response is open to interpretation. The question is how to modify a procedure that may require both a specific questioning and an answering time each day.

The implications of a time lag between phosphate enrichment and growth reaction are considerable, particularly if an endogenous rhythm controls the phase and amplitude of the growth maximum. Stability of the phytoplankton community becomes a property of the taxa that dominate the assemblage and only incidentally relates to qualities of the external environment such as the phase and amplitude of rhythms in nutrient concentration (*cf.* Ganf, 1974). The presence of a time lag may allow the assemblage to appear to exist in a nutrient limited, but nutrient sufficient, condition as noted by Peterson *et al.* (1974). The obvious next step is to identify and culture taxa with time-specific demands and responses to external nutrient concentrations.

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SUMMARY

Experiments were carried out to determine if photosynthetic rates of phytoplankton were nutrient-limited in a temperate lake and in an arctic pond. They were also designed to determine if daily or seasonal patterns existed.

Photosynthetic rates were measured under defined light intensities and temperatures, at various concentrations of sodium phosphate. Daily patterns were determined with 5 or 7 measurements made over a 24 to 30 hour interval.

Photosynthetic rates were stimulated by the addition of phosphate to samples of water from Lake George, New York. They were stimulated on only some of the sampling dates during one year of survey. When phosphate was not limiting, neither were other essential nutrients, with one exception. Later work, in the arctic, suggested that a negative result should not be interpreted as a non-limiting situation, however.

Arctic phytoplankton show strong daily oscillations in photosynthetic rates. The daily maximum occurred most frequently in the early evening, at 1800; that is, at the supper hour. Manipulation of the samples was necessary to evoke the response.

The addition of phosphate, or storage of the samples in low light intensity, if done early in the day, was an adequate treatment to evoke the daily maximum. The synchrony of the peak under a variety of treatments was argument for the involvement of an endogenous rhythm rather than a direct environmental reaction.

The lag in reaction to nutrient stimulation is viewed as part of a mechanism to promote stability of the phytoplankton community and an obstacle in the interpretation of negative results from short-term nutrient bioassays.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

Abstracts are arranged alphabetically by first author. Author and subject references also will be found in the regular volume index, appearing in the December issue.

GENERAL SCIENTIFIC MEETINGS

August 19–22, 1975

Membrane changes associated with the early stages of cell fusion in Tetrahymena. W. Steven Adair and Jean-Paul Revel.

When complementary mating types of the ciliated protozoan *Tetrahymena* are transferred from the medium in which they are grown to a simple inorganic salt solution, a number of physiological changes occur. The cells become arrested in the Gl phase of the cell cycle and develop a competence to pair. When competent cells are mixed and examined at various times using the freeze-fracture technique, conspicuous arrays of intramembranous particles are observed in the oral region at times which correlate roughly with the onset of cell fusion. Oral regions were examined at a number of times after mixing and scored for the presence or absence of membrane particle arrays. Of the 55 oral regions examined from samples taken up to and including 1 hour after mixing (pre-fusion) none were found to have the arrays. This is in sharp contrast to later times (post-fusion) where 31 of the 41 oral regions examined contained the arrays. Intermediate times are now being examined closely.

Individual particles measure approximately 130 Å in diameter and are often present as long linear arrays of 5–6 rows. The arrays show a sharp localization to the oral region of the cell, specifically in the area of the attachment plate (where fusion occurs) and extend a short way along the meridional ridges. The presence of such arrays at a time when fusion is observed and their localization to the site where fusion will occur, raises the possibility that they may represent a differentiation of the plasma membrane of these cells which is associated with membrane fusion. In light of the recent demonstration of a similar involvement of intramembranous particles during myoblast fusion and virus induced red blood cell fusion, it is suggested that such membrane differentiations might represent a general phenomenon associated with membrane fusion.

Ultrastructure of the extracellular matrix around dissociated sponge cells, Microciona prolifera. James M. Anderson.

The species-specific reaggregation of marine sponges requires the presence on cell surfaces of a high molecular weight proteoglycan complex called aggregation factor. The present study was undertaken in order to determine if other extracellular matrix materials exist around dissociated sponge cells which might also play a role in selective adhesion. Scanning and transmission electron microscopy and the freeze-etch technique reveal an extensive matrix which makes unknown interactions with aggregation factor and perhaps has no connection with cell reaggregation. All observations were on cells fixed on glass surfaces after settling for various times up to one hour. Etched cell outer surfaces are frequently covered with a dense feltwork of 4 nm diameter fibrils of indeterminant length. The surface coat can be tens of nanometers thick, is nonuniformly distributed on the soma, and is usually thickest on microvilli. What appear as coated microvilli in freeze-etching might also be interpreted as extracellular assemblages of the 4 nm fibrils. Scanning and transmission electron microscopes reveal a complex net of extracellular fibers around single and reaggregated cells. Various sizes are seen; the largest are approximately 0.1 microns in diameter. They adhere to glass and can extend for many microns between cells, branching and refusing in a web-like fashion.

If the aggregation factor is removed by washing in divalent-cation free sea water, the sponge tissue disaggregates into single cells. Under the same conditions both fiber classes persist, although extreme washing will remove most of the 4 nm fibrils. The connection between the reaggregation process and the extracellular materials described is unresolved. Transmission electron microscopy of intact sponge shows a dense connective tissue of fibers called spongin. By analogy with collagen assembly, it is tempting to describe the smaller surface fibrils as precursors in the formation of the extracellular matrix. This matrix, which is readily established around dissociated cells may then be spongin.

Energy coupling mediated by a brush border Na, K- ATPase in the active asborption of glycine in toadfish intestine, in vivo. DEREK BARKALOW AND A. FARMANFARMAIAN.

In vivo experiments in this laboratory have suggested that the active absorption of amino acids and sugars by intestine is not indirectly coupled to ATP hydrolysis via Na^+ or K^+ gradients. Instead, evidence has pointed toward a direct coupling of ATP hydrolysis to glycine transport. The present experiments were designed to further elucidate the nature of this energy coupling in toadfish intestine.

The addition of 10 mM ATP to a test solution of glycine in fish ringer placed in the lumen of an intact midgut sac caused a reduction in the net uptake of glycine by 35%. 10 mM ADP also caused a similar reduction; 10 mM AMP had no effect on net glycine uptake. It is postulated that this effect of ATP is due to a phosphorylation of the glycine "carrier" resulting either in an allosteric reduction of affinity of the carrier for glycine and/or in a reduction of the number of sites available for glycine transport. Under normal conditions ATP is only available at the intracellular interface of the membrane, creating a membrane asymmetry which could cause the accumulation transport of glycine.

The reduction of net glycine absorption by luminal ATP has been shown not to be caused by a chelating action of ATP for Ca^{++} or Mg^{++}. Approximately tenfold increase in Ca or Mg did not abolish the ATP effect. The role of a Ca, Mg-stimulated ATPase in glycine absorption is also ruled out by the data. In the absence of Ca or Mg in the test solution inhibition of glycine transport by ATP is undisturbed. However, a Na, K-stimulated ATPase involvement is implicated. A kinetic series of experiments was designed to test the interaction of Na in the incubation solution with luminal 10 mM ATP. When the initial incubation solution contained no Na (mannitol substituted), luminal ATP had no effect on net glycine absorption. As Na in the incubation solution was increased, the inhibition caused by luminal ATP gradually became evident, and at 100 mM Na, the full 35% reduction was seen. Preliminary evidence has also suggested a K^+ interaction with this ATPase associated with glycine transport. When 0.5 mM ouabain is included in the luminal solution the ATP effect on glycine transport is abolished.

The Na-ATP interaction is postulated to be due to the involvement of a brush border Na, K-ATPase mediating the energy coupling of active glycine absorption to ATP hydrolysis.

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A comparison of adaptive strategies of predation among naticid gastropods. CARL J. BERG, JR.

Predatory behavior of three species of naticid gastropods, *Lunatia heros, L. triseriata,* and *Polinices duplicatus,* was observed in the laboratory. Naticids capture, manipulate, and position prey for boring using their large foot. Borehole diameter is highly correlated with predator shell height. Furthermore, the boreholes of *L. heros* are significantly larger than those of *P. duplicatus*. Each naticid species bores through the shell of each prey species in a distinct area. Variability in borehole position was examined at species and individual levels. Each individual snail has its own characteristic pattern of borehole distribution which, taken collectively, form a slightly more variable species-specific pattern. Rearing studies with *L. triseriata* have shown that newly hatched snails capture, manipulate, and bore into prey in a species typical fashion and area. For all species, boreholes are more widely distributed on *Mercenaria mercenaria* than on *Mya arenaria,* and along the dorsal-ventral axis than along the anterior-posterior axis.

The adaptive significance of the restricted borehole positions is attributed to the relative thinness of the shell in the areas bored and the gross external morphology of the prey. The prey's internal anatomy seems less important in determining borehole position, especially since it is all eventually consumed. In general, snails of the two species of *Lunatia* exhibit similar predatory behavior patterns and strategies, which differ from those of *P. duplicatus.* Both genera appear to have the same prey preferences. Snails of the *Lunatia* species select larger prey, bore larger holes through thicker areas of the shells, and are more variable in borehole position than snails of *P. duplicatus.* Differences in borehole position relate to behavioral differences in prey capture and handling. Species-specific differences in borehole size and position may be used to indirectly assess predator-prey interactions in both modern and fossil faunal assemblages.

This work was supported by a research scholarship from the M.B.L.

Dichroism, birefringence and structural twist in polarized-light detectors of insects. GARY D. BERNARD AND RÜDIGER WEHNER.

In the dorsal part of the worker bee's eye two types of rhabdoms occur: 1) straight rhabdoms containing nine rhabdomeres over their entire lengths; and 2) twisted rhabdoms consisting of eight long rhabdomeres and a short proximal ninth rhabdomere. In the latter type, half of the rhabdoms twist clockwise and half counterclockwise, both with a twist rate of one degree per micron. The ninth cell and two of the overlying long twisted cells are ultraviolet receptors which are engaged in polarized-light detection.

Our optical analysis assumes that the principal axes for both birefringence and dichroism of a rhabdomere is the microvillar direction, and that all light guided by a rhabdom is carried within the volume of the rhabdom and by only the first mode. At a given level in the rhabdom this mode can be resolved into two mutually perpendicular, linearly polarized waves. Each has a longitudinal attenuation constant that is independent of both amplitude and phase of the other.

We conclude that: 1) polarizational sensitivity (PS) of a straight retinular cell is independent of birefringence; 2) PS of a long, twisted UV cell is unity for low birefringence and increases with increasing birefringence; 3) PS of the short, ninth cell is high for low birefringence (as birefringence increases, PS first decreases to a minimum PS-value of near unity before increasing to high values); and 4) for low birefringence the two types of short, ninth cells are maximally sensitive at planes that differ by sixty degrees.

These data suggest a sufficient minimum model for E-vector detection involving the UV receptors of only two twisted ommatidia: two ninth cells of different twist type act as polarization sensitive channels and the long UV cell(s) as a polarization insensitive channel.

Arsenazo III, an indicator for rapid changes of intracellular ionized calcium in squid giant axons. J. E. BROWN, L. B. COHEN, P. DEWEER, L. H. PINTO, W. N. ROSS AND B. M. SALZBERG.

When arsenazo III (mol wt 757) binds calcium, the absorption increases at long wavelengths and decreases at short wavelengths; the maximum increase in absorption is at 660 nm. This absorption increase can be used to detect small changes of Ca in the presence of high concentrations of Mg. Cleaned giant axons of *Loligo* were injected with the potassium salt of arsenazo III to 0.5 mM; 0.5 mM dye did not appreciably alter the ouabain sensitive outflux of ^{22}Na. Injected axons having greater than 400 m diameter were voltage-clamped; voltage-dependent membrane currents and absorption at 660 nm through a 1.5 mm space-clamped segment of axon were measured simultaneously. By averaging over many trials, an absorption increase at 660 nm in response to depolarizing voltage-clamp steps was detected. The signal was greatly reduced when Ca_{out} was reduced from 112 mM to 10 mM. This change in optical signal was reversed in sign at 530 nm and could not be attributed to changes in light-scattering, nor to voltage-dependent absorption changes of membrane-bound dye. We conclude the change in absorption detected the very small changes of Ca_{in} associated with Ca entry during depolarizing voltage-clamp steps. The time constant of the absorption change at 660 nm was approximately 400 μsec. In axons injected to 0.5 mM arsenazo III plus 8 mM tetraethylammonium ion, the Ca entry elicited by the second of a pair voltage steps, timed so that the early inward current was inactivated, was about 20% that elicited by a single voltage step. Similarly, the addition of 100 nM TTX reduced the Ca entry about 80%. These findings confirm the identification (by the use of aequorin) of an early phase of Ca entry associated with the early inward Na current elicited by depolarizing voltage steps.

The block to polyspermy in sea urchin fertilization: an analysis with agents affecting the cell surface. E. WILLIAM BYRD, JR.

Sea urchin eggs remain monospermic during fertilization by excluding the entry of all but one sperm. There have been two mechanisms proposed to account for the block to polyspermy: a fast, incomplete block which inhibits further sperm entry after the first successful contact of the fertilizing sperm, and a slower, complete block initiated by the cortical reaction. Recent work on *Stronglyocentrotus purpuratus* has shown that there is no fast block to polyspermy and that untreated eggs can be made highly polyspermic. By utilizing spermicidal agents the number of successful sperm-egg fusions at various times after insemination under polyspermic conditions can be measured. Several polyspermic agents have been investigated to clarify their mechanism of action in *Lytechinus pictus.* These agents can be divided into two major groups: 1) agents which enhance sperm-egg fusion rates prior to cortical granule breakdown (NH_4, nicotine), 2) agents which alter the vitelline layer (dithiothreitol) or inhibit the normal elevation of the fertilization membrane (soybean trypsin inhibitor). DTT works by increasing the rate of sperm-egg fusion prior to normal cortical granule breakdown. SBTI treated eggs have the same sperm-egg fusion rate prior to normal cortical granule breakdown, but continue to have successful sperm fusions after this event. Both groups of treated eggs can also be reinseminated after control cortical granule breakdown.

Observations on mating behavior in Limulus polyphemus. COLLEEN M. CAVANAUGH.

The American horseshoe crab, *Limulus polyphemus.* found only along the shores of the Atlantic coast from Maine to the Yucatan, migrates to the shallow waters of bays, estuaries and sounds in the spring to spawn. Fertilization is external. The female burrows into the sand and deposits her eggs, then the male, clasped on to the rear of her carapace by a modified first pair of walking legs, fertilizes them.

Spawning in *Limulus polyphemus* was observed in May and June, 1975, on the southern shore of the Mashnee Dike, Cape Cod, Massachusetts. Spawning occurred daily throughout May and into the first week of June, regardless of the moon phase, with the population density decreasing after June 6, 1975. *Limulus* were not found spawning by the end of June, nor during July and August. Spawning occurs only in the water and only after dark, mid to high tide, ebb or flood, with nests built close to the high water line; no animals were present in the intertidal area during daylight hours.

An average of ten males to one female was observed. Females never occurred alone. Usually one, but occasionally two or three males were attached to the back of the larger females. The attached males were neither noticeably larger nor smaller than the solitary males, based on carapace length and width measurements. Unattached males clustered about burrowing pairs, attempting to move under the attached male, spearing one another with their telsons in the process. Attached males were never dislodged by such activity. Whether unattached males also released sperm over the eggs was not determined.

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Potassium exchange in unfertilized sea urchin (Arbacia punctulata) *eggs.* EDWARD L. CHAMBERS.

A suspension of unfertilized eggs (0.05%) in sea water containing 0.05% streptomycin sulfate was divided equally between beakers A and B, and each stirred at 10 rpm, 20 ± 0.05° C.

At 0 time ^{42}K was added to A. After 18 h eggs in A were washed free of external isotope by decantation, simultaneously ^{42}K was added to B. Samples were removed from A and B over the next 18 h, centrifuged once, supernatant discarded, and ^{42}K, K and P content measured with corrections for interstitial fluid. Because of the highly dilute egg suspension the external [K] (8.9 mM), [^{42}K], and pH (8.3) remained constant; also ^{42}K entering the sea water from the eggs in A could be neglected. At all times egg samples removed for insemination developed to normal plutei. The egg [K] remained constant within experimental error at 204 μm K per ml eggs (range of ± 2 μm K). The flux computed from the initial rate of ^{42}K uptake was 2.7 pm K cm^{-2} sec^{-1}, and only 40% of the K in the eggs exchanged in 18 h. The fraction of unexchanged K at time, t, was computed from the ^{42}K loss data by dividing the egg specific activity (S.A.) by the value at 0 time, and from the uptake data by subtracting from 1 the product of the egg S.A. and the reciprocal of the sea water S.A. Plots of the natural logarithm of the fraction K unexchanged against time for both sets of data were curved, the slope of each curve decreasing 3-fold over an 18 h period (decreasing rate coefficient); moreover at any given time the slope of the curve for the ^{42}K loss data was 1.9 times greater than the slope of the curve obtained from the ^{42}K uptake data. These results are indicative of non-homogeneity of K in the unfertilized sea urchin egg.

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Effect of some inhibitors of proteolytic enzymes on maturation of starfish oocytes. THEODORE G. CLARK AND HARUO KANATANI.

The effect of two protease inhibitors on the meiotic maturation of starfish oocytes was examined. The inhibitors leupeptin and antipain, small peptide aldehydes of microbial origin, were used in this study. Oocytes from two species of starfish, *Asterias forebesi* and *Patiria miniata,* were isolated free from their surrounding follicle cells and incubated in artificial sea water containing a range of concentrations of leupeptin for one hour. After pre-incubation the oocytes were transferred into artificial sea water containing equivalent amounts of the inhibitor and 2×10^{-7} M 1-methyladenine to induce maturation. The percentage of germinal vesicle breakdown was then determined. Leupeptin was able to completely inhibit oocyte maturation in *Asterias* at a concentration of 1 mg/ml. In oocytes of *Patiria,* 100% inhibition of maturation occurred above 0.5 mg/ml. Using the same experimental procedure it was found that antipain also completely inhibits the maturation of *Asterias* oocytes at a concentration of 1 mg/ml. In order to determine whether the inhibition was simply due to toxic effects, after treatment with inhibitor, oocytes were removed and incubated in two changes of artificial sea water for a total of 45 minutes. The oocytes were then treated with 1-methyladenine and subsequently examined for germinal vesicle breakdown. In every case studied, it was found that the effect of the inhibitors was completely reversible.

This study was performed in the Reproductive Biology Research Program at the Marine Biological Laboratory (NSF grant BMS 75-03864). Supported in part by the Population Council grant M 75.06C.

Spontaneous maturation of isolated ovarian follicles from the starfish (Asterias forbesi): *Interaction of p*H, *Ca^{+2} and follicle cells.* J. G. CLOUD AND A. W. SCHUETZ.

During the normal spawning season of the starfish (*Asterias forebesi*) a large proportion of the ovarian follicles mature spontaneously when released from the ovary into sea water. Studies were carried out to elucidate the interactions of pH, Ca^{+2} and follicle cells in spontaneous maturation of the oocyte. Release of follicles from the ovary into acidic sea water (pH 4.7–5.4) resulted in inhibition of spontaneous maturation of the oocytes and maintenance of follicular structure. However, oocytes in acidic sea water were capable of undergoing maturation as evidenced by induction of germinal vesicle breakdown with 1-methyladenine (1 μg/ml) at low pH. Inhibition of spontaneous maturation in acidic sea water was reversible; inhibited follicles matured spontaneously when transferred into sea water at pH 8. Reversibility of inhibition decreased as incubation time in acidic sea water increased. Incidence of spontaneous maturation of follicles in sea water within the pH range of 4 to 7 increased as pH increased but was not different in media of pH 7 to 9. Follicles isolated in sea water (pH 5) did not spontaneously mature when transferred into calcium-free sea water at pH 8; the calcium concentration in sea water required to elicit a maximum response (per cent spontaneous maturation) decreased as pH increased. Oocytes devoid of their follicle cells did not mature spontaneously at any calcium concentration or pH. To examine whether spontaneous maturation of the oocyte in an individual follicle was due to release of meiosis-inducing substance (MIS), follicles isolated in sea water at pH 5 were transferred to sea water (pH 8) adjacent to defolliculated oocytes. Both defolliculated and intrafollicular oocytes matured demonstrating that MIS was released into the surrounding media in sufficient quantity to induce maturation of a number of oocytes. These results suggest that pH of the follicular environment modifies a Ca^{+2}-dependent release of MIS from the follicle cells.

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Site of production of maturation-inducing substance in the starfish testis. ROGER C. COCHRAN AND HARUO KANATANI.

In starfish ovary, a maturation-inducing substance (MIS), which induces germinal vesicle breakdown (GVBD) and spawning, is produced by follicle cells under the influence of radial nerve factor (RNF). Since starfish testis is also known to produce MIS in response to RNF, the site of production of MIS in the testis of *Asterias forbesi* was investigated. Testes were isolated in artificial sea water (ASW), and cut into small fragments. These were teased with fine forceps and left to stand 5 minutes to obtain a cell suspension, and a testis-wall precipitate. The suspension was centrifuged at 4430 *g* for 10 minutes. The resultant pellet and the testis-wall precipitate were each placed in equal volumes of ASW and incubated for 3 hr (22° C) with and without crude RNF (250 μg of lyophilized radial nerves of *Asterina pectinifera* per ml ASW). The incubates were then centrifuged (27,750 *g*, 20 min), and MIS activity of the supernatants was examined as GVBD of isolated *Asterias* oocytes. MIS activity was found only in the cell suspension incubated with RNF. Differential sedimentation of the cell suspension, which contained spermatozoa and other cells, was performed at 1 *g* to obtain a fraction of MIS-producing cells. The cell suspension (50 ml) was layered on 1200 ml of 3%–6% sucrose gradient in ASW in a Plexiglass cylinder. After standing for 5 hr at 2° C, the liquid was drained into 80 ml fractions which were centrifuged (4430 *g*, 10 min). The cell pellets obtained were resuspended in 2 ml of ASW, divided into 2 parts, and incubated with and without RNF (250 μg/ml) for 3 hr at 22° C. After centrifugation, the supernatants were assayed for MIS activity. Histological examination revealed interstitial cells in those RNF incubated fractions which had MIS activity. The corresponding fractions incubated with ASW had ⅓ the MIS activity. No other fractions either with or without RNF had any ability to cause measurable GVBD.

This study was performed in the Reproductive Biology Research Program at the Marine Biological Laboratory (NSF grant BMS 75–03864). Supported in part by the Population Council grant M 75.06C.

Urease from the lugworm Arenicola cristata. LYNN COOLEY, DANA R. CRAWFORD AND STEPHEN H. BISHOP.

Urea is a product of arginine and purine catabolism. In an organism with urease (urea aminodhydrolase, E.C. 3.5.1.5.), urea can serve as a source of metabolic ammonia. Lugworm gut, starting from behind the pharynx, was removed and washed. The tissue was homogenized and the brei centrifuged. Urease was demonstrated in the supernant fraction and was subsequently purified seventy-fold by chromatography with DEAE-cellulose and Sephadex G-150. The apparent molecular weight determined by gel filtration was 200,000. The K_m for urea varied with pH: it was high at pH 6.0 (2.4 mM) and low at pH 9.0 (0.2 mM); at the optimal pH for activity (pH 7–8), the apparent K_m was 0.34–0.38 mM. The V_{max} was highest at the optimal pH for activity. Of four inhibitors tested, acetohydroxamic acid (2×10^{-3} M) was most potent, inactivating 98 per cent of enzyme activity. The sulfhydryl reagents, N-ethylmaleimide (4×10^{-3} M) and dithiodinitrobenzoate (2×10^{-3} M), were less effective antagonists, reducing the activity by 77 and 57 per cent respectively. The characteristic properties of the urease from *Arenicola cristata* gut tissue—low K_m; variation of K_m with pH; pH optimum; molecular weight; and sensitivity to sulhydryl reagents—distinguish it from other ureases found in cestodes, land snails, bacteria and plants.

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Effect of gravity and exercise on the heart rate and blood pressure of bluefish (Pomotamus saltatrix). A. B. DuBois, P. Carniol, and R. D. B. Morris.

Pressure measured in the ventral aorta of 18 bluefish averaged 108/55 cm H_2O (s.d. 26/17), heart rate 43/min (s.d. 17). 17 were tilted head up in air for 1 min. Blood pressure was referred to a level opposite the heart. The mean BP horizontal was 96/50. At 10° it increased by 7/15 cm H_2O, at 20° by 7/15, at 30° by 10/18, signifying overshoot. At 40° the change of 3/9, and at 50° of 1/9, were not significant. Atropine sulfate (0.01 to 0.02 mg/kg) reduced the overshoot measured at 10° and 30° by 6 cm H_2O. Epinephrine in a 1.2 kg fish raised the resting blood pressure and heart rate as follows: Control BP 108/68, rate 33; 0.4 μg, 160/72, rate 32; 2 μg, 185/75, rate 39; 4 μg, 200/90, rate 52; and 10 μg, 210/105, rate 63. Swimming at 1–2 mph increased the BP in 4 fish from 105/81, rate 81, at rest to 117/96, rate 103, swimming. After atropine, the pressure was 111/91, rate 112, at rest, and 110/92, rate 113, swimming. After recovery from atropine, the pressure was 103/71, rate 99, at rest, and 110/86, rate 110, while swimming. Bluefish have a high resting blood pressure which increases still more with exercise or tilting. At rest, the heart rate and diastolic pressure are reduced by parasympathetic inhibition. This is released by the administration of atropine, abolishing the response to exercise, and reducing the response to tilting. The overshoot of blood pressure seen during tilting suggests the existence of partial circulatory compensation for gravity despite no previous hydrostatic stress. This reaction may have evolved in response to forward acceleration and hydrodynamic stress.

Uptake of glycine by the coccolithophorid Cricosphaera carterae, *a unicellular marine alga.* Diana Duckworth and A. Farmanafarmaian.

The coccolithophorid *Cricosphaera carterae* is shown to be capable of accumulating glycine against a concentration gradient. Free amino acids are found in sea water in concentrations of nmoles/ml; suggestions have been made that these free amino acids are nutrients for the growth of phytoplankton, especially under low nitrate conditions. Populations of *C. carterae* were grown at light intensities of 600 foot candles in a chemically defined artificial seawater medium. Cells were harvested during logarithmic growth. This organism probably possesses a synthetic pathway for glycine since there is no amino acid requirement for growth. Time course experiments at 3.3, 5, 6.7, 10, and 20 mM glycine exhibit linear uptake over 10 minutes and reach a steady state by 20 minutes. The relationship between net flux and substrate concentration appears to follow a sinusoidal curve with a first order region between 3 and 10 mM glycine and zero order transfer between 15 and 20 mM glycine. Thus uptake of glycine is carrier mediated. Terminal distributions of glycine partitioned between intracellular and extracellular compartments show that glycine is accumulated against a concentration gradient within 5 to 10 minutes at 3.3 and 6.7 mM glycine. These experiments were carried out in the presence of nitrate and under reduced light intensity. Glycine and amino acid transport in general may be important in the metabolism and productivity of this and other marine algae under aphotic conditions. Evolution of such active transport mechanisms may have conferred an advantage upon extant coccolithophorid species helping them to survive the faunal and floral crises that paralleled the Late Cretaceous fall in sea level.

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Morphologic differentiation can approximately parallel genetic differentiation in in the ectoproct Schizoporella errata *in coastal populations from Woods Hole to Orleans.* Alan R. Dutton and Thomas J. M. Schopf.

The question was asked whether changers in morphologic characters would bear a direct relationship to a known cline in genetic differentiation. Avicularium length, zooid width, and orifice length and width were measured in *Schizoporella.* Part of these same colonies were electrophoresed and gene frequencies determined in each of 8 populations for leucine amino peptidase and glutamate oxalate transaminase in standard ways.

Change in avicularium length parallels the cline in allele frequencies, but the other characters are more conservative and change little from place to place. Mean avicularium length changes from 0.126 mm at Woods Hole to 0.092 mm 80 km distant at Meeting House Pond, Orleans, Massachusetts. This change in length is significantly correlated with distance from Woods Hole ($r > 0.9$), and with temperature at the warmest time of the year ($r \simeq 0.8$). Allele frequency for LAP is also significantly correlated with temperature at warmest time of year ($r \simeq 0.9$), but is correlated with distance from Woods Hole only at the 0.10 level of significance ($r > 0.6$). Temperature and distance can only generally be related because nearly enclosed embayments and open waters may be near to each other but the bays heat up much more readily. In any event, the morphologic character which changed the most from one locality to the next generally tracts genetic differentiation as indicated by changes in frequencies of marker genes. This appears to be the first study which compares both morphologic *and* genetic differentiation in the same marine species.

Supported by NSF grant GB-30870 to Schopf.

Local membrane currents in Limulus *ventral photoreceptors.* ALAN FEIN AND J. SHERWOOD CHARLTON.

The ventral photoreceptors are located on the so-called lateral "olfactory" nerve which was removed from the animal, desheathed and pinned to the bottom of a chamber with a transparent base. These large photoreceptors (typically 50×150 μm in cross section) were stimulated by a small movable spot of light (nominally 30 μm diameter) that could be positioned anywhere on the photoreceptor. Under visual control (compound microscope 400×) a portion of a single photoreceptor was gently drawn into a glass suction electrode. Then the same cell was impaled by a KCl filled micropipette. Thus we were able to measure simultaneously the extracellular currents using the suction electrode and the receptor transmembrane potential using the intracellular pipette. When the region of the photoreceptor inside the suction electrode was illuminated, we measured a flow of current from the bath into the suction electrode. The path was completed when the current flowed into the illuminated region of the cell inside the suction electrode and out of the cell through the unilluminated regions outside the suction electrode. When the region of the photoreceptor outside the suction electrode was illuminated the extracellular current reversed direction. In both cases the intracellular recordings of the receptor transmembrane potential showed that the receptor current depolarized the cell. These results inicate that localized illumination of these photoreceptors leads to a flow of inward membrane current into the region of illumination.

An identified serotonergic input has reciprocal effects on two electrically coupled motoneurons in the terrestrial slug, Limax maximus. ALAN GELPERIN.

Two identified motoneurons, both of which are activated during the protraction phase of feeding, receive synaptic activation of opposite sign from the serotonergic metacerebral giant cells (MCGC). One motoneuron is an autoactive bursting cell, the salivary nerve burster (SNB). Bursts in SNB can be reset by intracellular current pulses and produce salivary duct muscle potentials and contractions. The other motoneuron, buccal cell 7 (B7), produces potentials in the supralateral radular tensor muscle which follow B7 spikes one-for-one at 20/sec. Intracellular recordings from B7 reveal 1 mV coupling potentials coincident with spikes in SNB. Subthreshold depolarizations in B7 drive bursts in SNB while hyperpolarizations in B7 block bursts in SNB. The effect of MCGC activity on B7-SNB coupling was determined while applying periodic subthreshold depolarizing pulses to B7 which reliably produced bursts in SNB. MCGC activity at rates ≤ 1 spikes/sec caused the subthreshold depolarizing pulse in B7 to become suprathreshold, producing several action potentials in B7. Simultaneously, the burst in SNB driven by B7 became shorter, delayed from the onset of depolarization in B7, and occasionally the SNB burst blocked entirely. During feeding the MCGC is active at low rates (≤ 1 spike/sec) and the SNB bursts during protraction, as judged by *in vitro* and *in vivo* recordings. The hypothesis currently under investigation suggests that the inhibitory effect of MCGC on SNB serves to enhance the phase locking of SNB to protractor motoneuron bursts by reducing SNB activity between protractor bursts and causing SNB bursts during protraction to be short and of high frequency.

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Studies on Woods Hole Tintinnida using scanning electron microscopy. KENNETH GOLD AND ELEANOR A. MORALES.

Loricae of *Stenosemella ventricosa, S. oliva, Tintinnopsis acuminata, T. dadayi, T. platensis, T. rapa,* and *Tintinnidium fluviatile* were examined by scanning electron microscopy (SEM) following air and/or critical point drying, in order to determine the origin and types of materials utilized in lorica building. Differences were observed with respect to the proportion of non-biogenic to biogenic materials utilized. *Stenosemella* spp. and *T. rapa* relied predominantly on quartz grains for lorica building; these species incorporated the silt-sized fragments directly into the lorica structure. The other species utilized far greater amounts of detrital material such as diatom and protozoan shell fragments and coccoliths, as well as grains and flakes of non-biological origin. The fragments appeared to adhere to an underlying matrix such as that seen in cultured specimens.

The range of particle sizes of both biogenic and non-biogenic origin was similar on most species, from < 1 to ~ 15 μm. *T. fluviatile,* an exception, took up entire diatom frustules; a maximum length of one such frustule was 36 μm. No obvious relationship was seen between the length of a species lorica and the sizes of the particles utilized. Certain specimens, however, did show size differences among particles according to the region of the lorica where they were found. As examples, the range of particle sizes surrounding the neck of *T. rapa* and *S. ventricosa* was < 1–4 μm, compared with particles up to 8 and 14 μm respectively elsewhere on the lorica.

There was good agreement between the appearances and sizes of particles utilized by the tintinnids *Stenosemella* spp., the arenaceous *Tintinnopsis* spp., and a foraminiferan from Sippewissett Marsh, possibly *Trochammina* sp. This observation adds emphasis to our earlier hypothesis that tintinnids such as *Stenosemella* produce their loricae in the sediments. The following species, not examined by SEM, were also encountered in Eel Pond and great Harbor between 9 June–20 August, 1975; *Tintinnopsis baltica, T. levigata, T. minuta, T. nucula, T. strigosa, T. sufflata, Helicostomella subulata, Metacylis* sp., *Tintinnus angustatus,* and *T. pectinis.*

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Photo-induced dichroism in a rhabdomeric photoreceptor: evidence for restricted rotation of pigment molecules. TIMOTHY H. GOLDSMITH AND RÜDIGER WEHNER.

Recent evidence indicates that rhodopsin molecules not only undergo translational diffusion but are also free to rotate in the planes of the disk membranes of rod outer segments. Both polarization sensitivity of arthropod retinular cells and measurements of dichroic absorption of isolated crustacean rhabdoms, on the other hand, indicate some preferential alignment of the chromophores with the axes of the microvilli, implying restrictions on the orientations that the pigment molecules can assume. If in rhabdoms the pigment molecules are unable to rotate in the tangent planes of the microvilli, it should be possible both to decrease the dichroic ratio by selective bleaching with light polarized parallel to the microvillar axes, and to increase the dichroic ratio with light polarized perpendicular to the microvilli. Using isolated rhabdoms of the crayfish *Procambarus* and *Orconectes,* such photo-induced dichroism has been observed in single layers of microvilli, studied with a laterally incident microbeam and a recording microspectrophotometer.

At pH 9 and in the presence of 0.25 M glutaraldehyde or alcohol-free formaldehyde, the normally stable metarhodopsin of crayfish photobleaches in several minutes. Photo-induced dichroism is observed, however, not only in the presence of glutaraldehyde, but also in formaldehyde. A quantitative theoretical description of photo-induced dichroism in microvilli has been developed for comparison. The magnitude of the observed photo-induced dichroism is less than theory predicts for random orientation of chromophores, consistent with the hypothesis that not all rotational positions are allowed. The photo-dichroism is greater in the presence of the cross-linking reagent glutaraldehyde than it is in formaldehyde, however, indicating that the molecules are normally free to wobble within some limiting angle with the microvillar axis.

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Synaptic connection between medial giant axons in the lobster, Homarus americanus. C. K. Govind and Fred Lang.

In astacurans such as lobsters and crayfish, a pair of medial giant (MG) interneurons mediate the rapid tail-flip escape reflex by activating the flexor muscles in the abdomen in response to rostral stimuli. In the lobster, *Homarus americanus,* we find a synaptic connection between the MGs which may be electrical in nature.

We used 4th stage lobsters (approximately 14 mm total length) in which the MG is the only "giant" element in the cord, the lateral giant axon having not yet hypertrophied. This permitted unequivocal identification of the extracellularly recorded MG spike. Suction electrodes were placed on either side of the 1st abdominal ganglia and one half of the cord was stimulated between the 5th and 6th abdominal ganglia. Under these conditions a rostrally traveling (antidromic) MG spike was recorded from the posterior to the anterior electrode followed by a caudally directed (orthodromic) spike which appeared, first in the anterior electrode and then in the posterior electrode. This showed a functional connection between the MGs which was viable in both directions. If both MGs were stimulated by increasing stimulus intensity only a summed spike was recorded in both electrodes. Similar results were obtained in adult lobsters. The addition of 5 mM manganese to the bathing medium did not affect the synapse but did block neuromuscular synapses. However, substitution of sodium propionate for sodium chloride in the bathing medium increased the latency of the return spike and evenutally blocked it. These experiments suggest an electrical coupling between the MGs in lobsters.

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The contribution of photosynthate to turgor pressure changes in a gas vacuolate blue-green alga. Neil G. Grant and A. E. Walsby.

When the planktonic blue-green alga *Anabaena flos-aquae* is transferred from a low (500 lux) to a high (4 klux) light intensity its cell turgor pressure increases. Previous investigations have shown that this increase is completely inhibited by DCMU and does not occur in the absence of carbon dioxide. Thus, the turgor rise is dependent on photosynthesis.

We have tested the hypothesis that the rise in turgor pressure is due to a rise in the pool of low molecular-weight photosynthetic products dissolving in the cell water, increasing the osmotic pressure of the cell fluids. The alga was transferred to a high light intensity and simultaneously supplied with ^{14}C-labelled bicarobonate of known specific activity. At intervals, samples were removed, and cell solubles were extracted in methanol. Those of low molecular weight were separated by gel filtration on Sephadex G-25. Assuming that these substances contained six C atoms on average, the osmotic pressure they generate when dissolved in the cell water (determined by the method of Triplett and Walsby, 1975, *Biol Bull.,* 149: 448) could be calculated.

Our measurements showed that in some instances the rise in labelled photosynthate could just account for the observed turgor pressure rise; however under other conditions (as when the alga is simultaneously transferred to fresh culture medium) the turgor rise can be as much as seven-fold higher, perhaps indicating the involvement of other mechanisms, such as a photosynthetically driven ion pump.

This work was supported by a grant, BMS–73–06800 from the National Science Foundation.

Conformational changes in chromatin of spermatozoa of two prosobranch molluscs (Goniobasis *and* Busycon). CATHERINE HENLEY.

Late spermatids and mature spermatozoa of some prosobranch molluscs have a structural instability in the orientation of their nuclei. In the case of *Goniobasis*, nuclei of *all* typical (eupyrene) late spermatids and mature spermatozoa, subjected to routine hydrolysis for the Feulgen reaction, have the long axes of their nuclei at 90° to the long axes of the cells, rather than coincident with them. The configuration suggests the designation of "hammerhead". In *Busycon*, under poorly understood conditions of drying, the nuclear regions of some, *but not all*, eupyrene late spermatids and mature spermatozoa are thrown into a striking helical configuration. Preliminary observations in collaboration with Dr. Shinya Inoué, suggest that the observed helical conformation of the nuclear regions in such dried material may be an exaggeration of the underlying molecular organization. When studied with polarization optics such nuclei have a complex helical substructure, with chromatin wound around a straight central component.

Spermiogenesis and spermatogenesis in both *Goniobasis* and *Busycon* may offer clues to such diverse conformational changes. The nuclei of young eupyrene *Gomiobasis* spermatids undergo nuclear condensation with an intermediate lamellar stage in which helical strands, 130–140 Å in diameter, can be demonstrated by use of the freeze-etch technique. Another aspect of conformational changes in chromatin is seen in spermatogenesis of non-functional atypical spermatozoa in this form, where the meiotic spindles become disorganized and release chromosome vesicles into the spermatocyte's cytoplasm. By electron microscopy of conventional sections, such vesicles have 40 Å globules embedded in an amorphous matrix. Apparent spindle microtubule remnants, with 30 Å protofibrils, are often attached to them. Information at the level of electron microscopy for comparable stages in *Busycon* is not yet available, but light microscopy of Feulgen preparations strongly suggests that a somewhat similar, although not identical, situation exists in this form as well.

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Structural changes at the "active zones" of synapses in stimulated Torpedo *electric organs.* J. E. HEUSER AND T. S. REESE.

Field stimulation of the brain stem of anesthetized torpedos during vascular perfusion with aldehyde fixatives produced changes in the structure of nerve terminals in their electric organs. In thin sections, vesicle-sized dimples and other deformations of the presynaptic plasmalemma occurred beneath clusters of synaptic vesicles. Since these plasmalemmal alterations were rare in unstimulated electric organs, they are thought to represent exocytotic discharge of synaptic vesicles resulting from the stimulation. Panoramic views of stimulated synapses with the freeze-fracture technique illustrated that the plasmalemmal deformations occurred in discrete patches which thus constitute the "active zones" for discharge of synaptic vesicles at this synapse.

Also common in these stimulated synapses were hemispherical bulges in the plasmalemma where synaptic vesicles pressed against it. Boyne, Bohan, and Williams (1974, *J. Cell Biol.*, **63**: 780) have proposed that this sort of intimate vesicle contact could be sufficient to allow transmitter discharge without exocytosis. However, our results with the freeze-fracture techniques have revealed that membrane particles are totally absent from both vesicle and plasma membranes at these contacts. Since such particles are abundant in highly permeable regions of the plasmalemma, such as gap junctions, this absence should discourage the idea that the bulging contacts are permeable to transmitter. It seems more likely that they are an artifactitious exaggeration of the type of contact that synaptic vesicles make with the plasmalemma at the beginning of exocytosis, which may require the exclusion of intervening membrane particles.

Rhodanese and DFPase in relation to isetheionate in squid nerve. FRANCIS C. G. HOSKIN AND ELLEN R. KORDIK.

The enzyme rhodanese (thiosulfate: cyanide sulfurtransferase) is usually obtained from mammalian liver. We now find it in relatively high concentration in squid (*Loligo*) nerve and hepatopancreas, and either absent or present only in lower concentration, in the nerves of *Spisula* and *Aplysia.* Thus for molluscan nerve we note a distribution parallel to that of another enzyme, DFPase (diisopropylphosphorofluoridate fluorohydrolase), and of the anion isethionate ($HOCH_2CH_2SO_3^-$). Rhodanese and DFPase, but not isethionate, are present in squid hepatopancreas. Isethionate is not synthesized by squid nerve from cysteine–U–^{14}C despite the active uptake of cysteine, its metabolism to cysteinesulfinate, hypotaurine, and taurine, the presence in axoplasm of isethionate at a concentration of 0.15 M, and what would seem an obvious pathway from cysteine to isethionate. Furthermore, isethionate is not synthesized by squid nerve from ^{35}S-cysteine although this label reveals that the latter is readily metabolized to $^{35}SO_4^=$. Isethionate is not found in squid blood, and it is not taken up by squid nerve. From these seemingly isolated facts and the paradox of isethionate presence but not formation, we offer the following speculation.

Cysteine or some similar isethionate precursor is metabolized by squid hepatopancreas to a more immediate precursor of isethionate. Rhodanese participates in this, uniting thiosulfate or a thiosulfate analogue with cyanide or a cyanide analogue. This formation of the C-S bond is known to be a function of rhodanese. This more immediate precursor is transported to squid nerve where, as needed, DFPase hydrolyzes the acid anhydride storage form, thus releasing isethionate. Arguments which run counter to this speculation include the presence of DFPase in squid hepatopancreas and rhodanese in squid nerve. Further experimentation may result in considerable modification of these ideas. Nevertheless, the distribution of these two enzymes and the anion appears significant.

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Purification of Microciona prolifera *aggregation factor.* TOM HUMPHREYS, WES YONEMOTO, SUSIE HUMPHREYS, AND DAVID ANDERSON.

The purification protocol is based on Henkart, Humphreys and Humphreys (1973, *Biochem.,* **12**: 3045) where *M. parthena* factor was found to be a large proteoglycan complex with a fiberous, sunburst configuration. Purification of *M. prolifera* factor, which is a similar sunburst complex, required more rapid processing to prevent aggregation of the sunbursts and a Ca^{++} gelation step to remove a linear fiberous polysaccharide contaminant. After 4 washes of 10 min at 0° C each in 20 volumes of Calcium and Magnesium free sea water (CMF-SW), the tissue was squeezed in bolting silk into 10 volumes of CMF-SW. The resultant cell suspension was shaken for 2 hours at 4° C and the supernatant clarified by sequential centrifugation at 1500 rpm for 5 min, 10,000 rpm for 15 min, and 30,000 rpm for 30 min. One fifth volume of 0.1 M $CaCl_2$ was added to the supernatant and it was shaken gently at 25° C for 2 hours. The gel which formed was removed by decanting and centrifugation for 3 min at 3000 rpm and dissolved in 20 volumes of CMF-SW for 6 hours at 4° C with gentle shaking. The dissolved gel was centrifuged at 30,000 rpm for 30 min and the supernatant then centrifuged at 40,000 rpm for 90 min. The clear, gelatinous pellet was resuspended in 4% of the original volume of the supernatant in CMF with 10^{-3} M $CaCl_2$ for 3 hours at 4° C and passed over a 3000 Å pore size controlled pore glass column. After purification by the gelation step it is important to maintain Ca^{++} levels at 10^{-3} M to prevent rapid loss of activity. The purified factor contained 1.3 μg protein and 0.6 μg polysaccharide per unit of activity. When visualized with the electron microscope the preparations were almost exclusively structures with sunbrust configurations. During the 1975 season 80 units of activity were obtained from one gram of sponge. 60 to 70% of the original activity was obtained from the column.

Factors involved in oocyte maturation in the polychaete, Chaetopterus pergamentaceus. SUSUMU IKEGAMI, TOKINDO S. OKADA AND SAMUEL S. KOIDE.

In the polychaete, *Chaetopterus pergamentaceus,* mechanical release of oocytes from the parapodia into natural sea water results in the "spontaneous" breakdown of the germinal vesicle. Oocytes will remain at this stage until fertilization takes place. Immature oocytes thoroughly washed with calcium-free sea water mature in natural sea water, but not in artificial, calcium-free, magnesium-free, or calcium and magnesium-free sea water.

Several methods to induce oocyte maturation using well-defined chemicals have been established in the present study: 1. Germinal vesicle breakdown was induced by the treatment of immature oocytes with KCl (60 mM) in artificial or magnesium-free sea water. The presence of calcium ions greater than 4 mM was necessary for inducing oocyte maturation in higher potassium concentration. Lillie's "differentiation without cleavage" was observed after this treatment; 2. Trypsin (0.3%) induced oocyte maturation in artificial sea water, but not in calcium-free sea water. Oocytes matured in this manner developed normally to the trochophore stage upon insemination; 3. Immature oocytes, treated with 0.3 mM $CaCl_2$ (pH 6.4) for less than 1 minute and then transferred to artificial sea water, underwent germinal vesicle breakdown. The vitelline coats of all of the oocytes were removed by this treatment. The oocytes aressted at first meiotic metaphase and upon insemination developed into trochophore; 4. Tetracaine (0.4 mM) induced oocyte maturation even in the absence of calcium ions. In artificial, calcium-free, or calcium and magnesium-free sea water, oocytes were arrested at first meiotic metaphase. Oocytes treated with tetracaine in magnesium-free sea water developed parthenogenetically to the 4- to 8-cell stage and ceased to cleave. Since tetracaine is known to release calcium ions from binding sites in human erythrocytes, it is conceivable that this drug may act in a similar manner by releasing bound calcium ions in *Chaetopterus* oocytes and that these ions may be involved in the induction of germinal vesicle breakdown.

Studies on the mechanism of the initiation of polypeptide synthesis in sea urchin embryos in vivo. JOSEPH ILAN AND JUDITH ILAN.

The incorporation of labeled methionine into nascent peptides was studied in sea urchin embryos. After *in vivo* labeling, the polysomes were isolated, and the nascent peptides were released as peptidyl-tRNA by treatment with EDTA. Peptidyl-tRNA was further purified on hydroxylapatite and was subjected to Edman degradation. When the polysomes were at a steady state condition, most of the labeled methionine was found in internal positions of the nascent peptides and only 10% at the NH_2-terminal. Inhibition of protein synthesis *in vivo* by puromycin or by NaF brought about dissociation of polysomes. During the recovery period after the inhibition, the system was partially synchronized for initiation. Under such conditions about 40% of the labeled methionine incorporated into the NH_2-terminal position at 2 min. This value decreased to 10% in 15 min, suggesting transient incorporation of methionine into NH_2-terminal *in vivo*. Control experiments were carried out with labeled alanine, glycine, lysine and serine. At 2 min and at 15 min, all showed less than 10% incorporation into the NH_2-terminal position.

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Crystal property of larval spicule of Arbacia punctulata. SHINYA INOUÉ AND KAYO OKAZAKI.

In echinoderm pluteus larva, a pair of birefringent spicules arise from the calcareous triradiate formed in the gastrular mesoderm. In polarized light, each triradiate and left and right spicule exhibit a single optic axis despite its complex, species-specific shape. The optic axis lies normal to the triradiate plane and along the spicule major axis. Besides an organic matrix (1% by weight), powder X-ray pattern identifies the spicule as a Mg-calcite.

In larval sea urchins grown in sea water containing 1/10 normal Ca and Mg, calcite-like faces appear at the triradiate and spicule tips. These faces lie precisely parallel to cleavage faces of pure calcite appropriately oriented. The calcite optic axis then lies normal to the triradiate plane. The spicule is thus structured as though carved from a single calcite crystal. However, scanning EM of isolated normal, fractured, N/10 HCl or EGTA etched spicules failed to display etch or fracture pits definitive for calcite. We therefore decorated isolated spicules with calcite microcrystals by exposure to N/10 $NaHCO_3$ plus N/10 $CaCl_2$. Polarized light revealed all microcrystals to grow with optic axes parallel to the spicule optic axis. Scanning EM revealed striking stacks of calcite crystals arranged with parallel faces on each spicule and triradiate. At spicule junctions, microcrystals of the left and right spicules were tightly interlocked. Since optic axes and faces of the decorating crystals lie parallel to those observable in the spicule, decorating crystals must have grown epitactically revealing axes of the submicroscopic crystals of the spicule invisible with scanning EM.

We conclude that despite their complex morphology, the pluteus spicule is built up of stacked Mg-calcite crystals whose crystallographic axes lie strictly parallel to each other.

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Biosynthesis of δ-aminolevulinic acid in Cyanidium caldarium *and other algae.* J. E. JURGENSON, S. I. BEALE, R. R. TROXLER, M. M. BARTOLF, M. P. FITZGERALD, J. RAMUS AND J. A. SCHIFF.

The first committed step in the porphyrin pathway in animals and photosynthetic bacteria is catalyzed by δ-aminolevulinc acid synthetase (ALAS) which condenses glycine (GLY) and succinyl CoA to form δ-aminolevulinic acid (ALA). ALAS activity has not been detected in plants. Recently, it has been shown that α-ketoglutarate (α-KG) and glutamate (GLU) are better precursors of ALA in higher plants than are GLY and succinic acid. This suggests that ALA in higher plants may arise via an alternate route. The present work was initiated to inquire into the existence of an alternate mode of ALA biosynthesis in algae.

Cyanidium caldarium is a unicellular rhodophyte which lacks photosynthetic pigments when grown heterotrophically in darkness but which synthesizes chlorophyll-a and phycocyanobilin (tetrapyrroles derived from ALA) when dark-grown cells are placed in the light. Levulinic acid (LEV) is a competitive inhibitor of ALA dehydratase, the second enzyme in the porphyrin pathway. Dark-grown *C. caldarium* cells incubated in medium containing 4 mM LEV in the light (1) synthesized 50% less chlorophyll-a and phycocyanobilin than controls, and (2) accumulated ALA in quantities exactly equivalent to that expected based on inhibition of pigment biosynthesis. This suggests that accumulated ALA was that destined for tetrapyrrole biosynthesis. This stoichiometry was not observed in comparable experiments with *Euglena gracilis, Poryhpridium aerugineum,* and *Synechococcus cedrorum.*

During a 4 hour incubation, α-KG and GLU were incorporated into ALA 2–3 times more efficiently than GLY–2–^{14}C and 30–50 times more efficiently than GLY–1–^{14}C. Radiolabeled ALA was chemically degraded with periodate to formaldehyde (C_5) and succinic acid (C_1–C_4) fragments. C_5 of ALA contained radioactivity predominantly from C_1 of α-KG and GLU. C_1–C_4 of ALA contained radioactivity largely from C_5 of α-KG and GLU. C_1 and C_2 of GLY were poorly and inefficiently incorporated into C_5 of ALA in *C. caldarium.* This shows that the carbon skeleton of α-KG and GLU are incorporated intact into ALA in this alga. It was concluded that ALA in *C. caldarium* is derived by a mechanism other than that involving GLY and succinyl CoA condensation by ALAS.

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Shifts in tidal and diurnal rhythms of color change of Uca pugnax *due to burrow location.* CHARLES J. KELLY JR.

The circadian rhythms of *Uca pugnax* with respect to diurnal and tidal related cycles of color change were analyzed. Two groups of crabs on three occasions were taken from the Great Sippewissett salt marsh in Woods Hole, Massachusetts, as the high tide came in and burrow inundation occurred. The crabs designated "low" were taken from burrows located 20–25 cm above the averaged low tide mark. "High" crabs were taken from burrows located 40–50 cm above low tide. The crabs were placed in a constant dark chamber and remained there for 24 hours after which half hour readings were taken on their level of chromatophore dispersion. The crabs were observed for a 25 hour period. The index of chromatophore dispersion used was a 1 to 5 scale, 1 being the lightest state and 5 the darkest. Intermediate levels of chromatophore dispersion were judged according to extent rather than magnitude of dispersion: *e.g.,* darkening of the legs and large cheliped preceeded full darkening of the thorax region.

The results of the study contradicted previous findings regarding differences in *Uca* rhythms associated with burrow location. It was observed that "low" crabs exhibit the more extreme paleness at night and darkness during the day, the typical diurnal rhythm. It was suggested that because "low" crabs spend more time in their burrows, they have less time to feed and, consequently, spend a larger proportion of their time on the beaches. As such, there is impetus for the "low" crabs to match the single beach sand color, as opposed to the several substrate colors with which the "high" crabs have to deal. The second result was that the tidal rhythm for both groups of crabs appeared as a bimodality which moved across the diurnal curve. It was shown to a statistically significant level that the tidal "blip" on the diurnal curve appeared as an accentuation of the paleness at night and dark coloration during the day. During the dusk and dawn "inflection points" when crab coloration was changing rapidly, the tidal biomodality was not statistically significant.

This study was performed in conjunction with the Marine Ecology course at the Marine Biological Laboratory.

Microtubule depolymerization in local regions of the mitotic spindle by Ca^{++} microinjection. Daniel P. Kiehart and Shinya Inoué.

Pressure microinjection was used to change the ionic milieu of the mitotic spindle *in vivo*. A modified Hiramoto system allowed injection of 1/1000 egg volume into first and second division eggs of *Asterias forbesi* and *Lytechinus variegatus*. One mM $CaCl_2$, injected at the spindle pole totally dissolved the aster and a minute portion of the spindle. A sharp transition between the region where spindle birefringence (BR) was abolished and where BR remained indicated the effect of injection to be very local indeed. In contrast to this general pattern, a graded response was occasionally observed. Spindle BR was locally reduced without totally disrupting the fiber. In either case, anaphase was completed in a qualitatively normal fashion. Injections of similar volumes of 1 mM $MgCl_2$, distilled water, or ten times greater volume of Wesson oil induced no change in spindle BR or morphology. Cells completed division and continued developing normally.

Spindle BR was reduced or abolished only with Ca injection right at the spindle pole. A thirty-fold higher concentration of $CaCl_2$ injected 23 μm from the spindle gave no effect—diffusion theory predicts a concentration of free Ca^{++} sufficient to depolymerize fibers in the spindle. Since the spindle lost no BR, we infer that Ca^{++} is not fully diffuseable and that the cell is apparently capable of so rapidly sequestering Ca^{++} that steep gradients of this ion can exist in the cytoplasm for short periods of time.

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Gamete release from isolated sea cucumber gonads by 1-methyladenine. Samuel S. Koide, Susumu Ikegami and Haruo Kanatani.

Gamete shedding is induced in starfish by treatment with either 1-methyladenine (1-Ma) or a hormonal peptide, gonad-stimulating substance (GSS), *in vitro* as well as *in vivo*. 1-Ma is known to be produced in starfish gonads under the influence of GSS.

We have found that isolated fragments of ovotestes of the sea cucumber, *Leptosynapta inhaerens*, shed spermatozoa immediately upon treatment with 1-Ma at concentrations higher than 6.5×10^{-7} M. However, immature oocytes which are connected with each other by a thick follicular envelope inside the fragments neither spawned nor matured under the influence of 1-Ma, and their germinal vesicle remained intact. However, with a few specimens of gonadal fragments containing mature oocytes, treatment with 1-Ma resulted in a release of these oocytes. Shedding of sperm or mature oocytes from the cut surface of the isolated gonads appear to result from the contraction of the gonadal wall in response to 1-Ma. In addition to 1-Ma, 1-ethyladenine was the only other effective agent in inducing sperm shedding at concentrations higher than 1.3×10^{-6} M. The following compounds had no effect at the concentration of 5×10^{-6} M: adenine, guanine, 6-methylaminopurine, 7-methyladenine, 7-methylguanine, N^6-dimethyladenine and 1-methyladenosine.

On the other hand, acetylcholine chloride (Ach) at higher than 5×10^{-6} M induced shedding of sperm. The sperm shedding action of both 1-Ma and Ach was completely inhibited in the presence of D-tubocurarine chloride at concentrations higher than 5×10^{-5} M. These gonadal fragments shed sperm even in the presence of tubocurarine when one drop of isoosmotic potassium chloride was subsequently added to 1 ml of medium in which they were incubated. These results suggest that in case of gamete shedding of *Leptosynapta,* 1-Ma seems to exert its effect by acting on the gonadal nerve cells or at the neuromuscular junctions.

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Sediment deposition and growth irregularities in Chamaecyparis thyoides *kettle bogs.* AIMLEE D. LADERMAN.

The aim of this portion of a White Cedar swamp ecosystem study is to gain clues to determinative factors in bog biotic succession by analysis of its geological, vegetational and meteorological past. The bogs lie in the knob and kettle moraine left by glaciation that receded about 10,000 years ago. Kettle water level is contiguous with ground-water levels. As they have neither feeder streams nor outlets, precipitation is the main water source. Sediment cores of two Falmouth, Massachusetts swamps, Cumloden and MBL-N, were obtained with the Redfield Precision Piston Peat Corer (2.5 cm i.d.). Cores were wrapped and frozen for C^{14} dating and palynological analysis. Wet/dry-weight ratios and pigment absorption spectra were determined. Age and growth rates of *C. thyoides* was determined by study of annual growth in 3 mm cores obtained with a forester's increment borer. Sediment boring revealed a sediment depth maximum of 4.6 m above glacial till. Predominant deposit of the cores is a dark red-brown peat containing many undecomposed plant shreds. Woody fragments are found throughout. Occasional layers of fine-grained paler sand reflect major storms that eroded surrounding sand into the basins. Spectrophotometry of the peat shows large quantities of pigment absorbing in the blue region, with a smaller 670 nm (phaeophytin) component. Sand yielded peaks at 420 and 670 nm characteristic of carotenoid and chlorophyll degradants. Water content varied greatly, with sand holding up to 88% and peat as little as 2% wet weight. Measurement of trees in Cumloden reveal cedars aged 68–118 years (91 yr mean), with diameter at breast height (dbh) widths of 13–40 cm (24.5 cm mean) and heights of 7.5–14 m (12 m mean). MBL-N trees are older: 141–152 years (144 yr mean), dbh 22–32 cm (27 cm mean) and heights of 11–14 m (11.5 m mean). There is no general relation between size and age. Growth rings of the past two decades are narrow; trees on hummocks show healthier growth than those in hollows. There is substantial evidence that swamp water levels have risen during the past twenty years. Vertical accretion of peat indicates a rising water table, which in turn reflects a regional rise in sea level. C^{14} dating of similar deposits indicates ages of 3,000–5,000 years for peat at 4.6 m.

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Sodium efflux from voltage clamped squid axons. DAVID LANDOWNE.

Axons were injected with ^{22}Na and mounted in a chamber which permitted voltage clamping and collection of efflux samples. At rest, in artificial sea water containing ouabain, about 0.1% of the ^{22}Na leaves the axon each minute. Impressing a 40 mV voltage clamp pulse thirty times per second increased the efflux approximately ten-fold. Larger depoliarizations produced more extra efflux. The extra efflux was blocked by external application of tetrodotoxin.

To test the "independence principle" the sodium in the external sea water was replaced with Tris. This altered the voltage clamp currents predictably, the inward "sodium" current became outward. In conflict with the independence principle the extra efflux dramatically dropped. Replacing sodium with dextrose or Mg-mannitol produced a similar effect. This indicates that sodium efflux is not independent of the ions in the external solution. The extra sodium efflux exhibits sodium-sodium exchange-like qualities.

These exchange-like qualities are complementary to the single file diffusion qualities of potassium fluxes. Both indicate that ionic movement through the membrane is unlike diffusion through free solution. As a basic assumption in deriving the constant field equation was free diffusion within the membrane, these results suggest caution in the use of this equation.

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Trophic relationships at lower and intermediate steps in a salt marsh detritus-based food web. JOHN J. LEE, JOHN H. TIETJEN AND CARMINE MASTROPAOLO.

Several different experimental approaches were focused on clarifying the trophic relationships of meiofauna and small macrofauna from salt marsh aufwuchs communities. In one series of experiments ^{32}P labeled algae and meiofauna were placed in dishes for 24 hr as potential food for *Paleomonetes pugio, Bittium alternatum, Fundulus heteroclitus, Capitella capitata, Nassarius obsoletus* and various species of haustoriid and gammorid amphipods. After feeding, the macrofauna were placed in clean dishes for an additional 24 hr before being sacrificed and counted in order to measure label transferred after digestion. In general the above animals ate both algae and mediofauna. The rates of ingestion of the food tested varied from 1×10^{-5}–1×10^{-2} g (dry weight) assimilated/g (dry weight) animal/day. *Nassarius obsoletus* and *Bittium alternatum* consumed $\sim 10\%$ of the algae offered ($\sim 10^7$ cells) per day and $\sim 3\%$ of the meiofauna ($\sim 10^4$ animals). *Paleomonetes pugio, Fundulus heteroclitus* and *Capitella capitata* ate less of the food tested. Small species of algae (e.g., *Nannochloris* sp) were not captured by the amphipods which otherwise consumed large quantities of algae and the largest quantities of meiofauna of any of the macrofauna tested.

Gnotobiotic culture experiments were used to study the potential effects of the grazing pressure exerted by the amphipods on the meiofauna. In one week 6 amphipods consumed almost all the meiofauna ($\sim 10^5$ animals) in a medium sized (250 ml) tissue culture flask. The population was reduced when even 2 amphipods were present in the flask. Sand added to the flasks provided some refuge for the meiofauna. The fecal pellets of the amphipods were examined microscopically. Algal and meiofaunal fragments, bacteria, and some apparently undigested diatoms were found in the fecal pellets of the amphipods. The evidence obtained suggests that amphipods may be important links in coupling meiofaunal productivity to higher trophic levels.

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The action of 3- and 4-aminopyridine on synaptic transmission in the squid giant synapse. R. LLINAS, K. WALTON AND V. BOHR.

The effects of 3- and 4-aminopyridine (3-AP, 4-AP) on transmission in the squid giant synapse were studied. Following bath application, 1, 5, and 30 mM 3-AP or 4-AP were found to slow the falling phase of the pre- and postsynaptic spikes and to reduce or abolish the delayed rectification of current pulses injected into the presynaptic terminal. These effects are presumed to be due to blockage of potassium conductance ($g_K{}^+$). However, this blockage of $g_K{}^+$ became incomplete when current injections produced transmembrane potentials a few mV positive inside. We also found that depolarizing pulses must be separated by at least 10 sec to obtain reproducible results. Nevertheless, after TTX and 40 mM (Ca^{2+})o typical calcium spikes were observed in the presynaptic terminal. These regenerative voltage changes were accompanied by release of transmitter as indicated by the postsynaptic response. In fact, the drugs did not signficantly after synaptic transmission. Prolonged presynaptic depolarization caused sustained transmitter release as observed by the postsynaptic response. This response was not markedly changed for up to two hours. With due attention to the voltage and frequency restrictions mentioned above, it is concluded that 3-AP and 4-AP can be used as $g_K{}^+$ blocking agents in most experiments concerning the depolarization release coupling system in the squid giant synapse.

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Subunit interactions of fish hemoglobins. CHARLES MARKOWITZ, DAVID BESWICK, DANIEL KIEHART, AND DENNIS POWERS.

Human hemoglobins undergo reversible dissociation into subunits. Subunit contacts between non-identical polypeptides are primarily hydrophobic while contacts between identical subunits are primarily polar. In this study, NaCl and urea were used as molecular probes of polar and apolar contacts, respectively. The methods used were sedimentation velocity, sedimentation equilibrium, and large boundary gel diffusion. Hemoglobins were prepared from dogfish (*Mustelus canis*), scup (*Stenotomus versicolor*), and man. Increasing concentrations of urea, up to 4 M, shifted the subunit equilibrium toward the dimers for human hemoglobin, while cartilaginous and bony fish hemoglobins were essentially unaffected by urea. Subunit dissociation was increasingly shifted toward the dimers for both human and teleost hemoglobins as a function of salt concentration, but the dogfish hemoglobin showed little change in 2 M and 4 M NaCl.

It was hypothesized that complementary electrostatic and hydrophobic forces were stabilizing the dogfish tetramer. Electrostatic interactions were favored upon urea addition while hydrophobic interactions were accentuated upon salt addition. Therefore, dogfish hemoglobin was tested in a solution containing both 2 M NaCl and 2 M urea. Subunit dissociation was strongly shifted toward the dimer, supporting the above hypothesis.

Urea accentuates the electrostatic interactions which appear to be important in the stabilization of terameric fish hemoglobins. This adaptive molecular strategy may explain in part the physiological resistance of cartilaginous fish hemoglobins to internal 0.5 M urea. In comparison to hydrophobic interactions, electrostatic forces are much less affected by temperature. Consequently, the protein bonding domains of poikilothermic organisms living in fluctuating thermal environments may show increased involvement of electrostatic interactions.

In general, one might predict that electrostatic interactions at bonding domains of protein will be accentuated in poikilothermic organisms living in fluctuating thermal environments such as the intertidal zone, while hydrophobic interactions will be accentuated in organisms living in constant thermal environments like the deep ocean.

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Temperature and salinity effects on Melampus bidentatus *Say with special reference to oxygen uptake rates and survival.* ROBERT F. MCMAHON AND W. D. RUSSELL-HUNTER.

The primitive pulmonate of the high salt marsh, *Melampus bidentatus* has a lung and no vestige of a ctenidium. It experiences short periods of submergence, variable salinity, and generally high and variable environmental temperatures.

Survival of total submergence at 20° C in specimens of *Melampus* was up to 3 days at salinities $>7.75‰$ and <2 days in FW (freshwater); at 10° C, >14 days above 7.75‰ and >9 days in FW. In stocks acclimated (2 weeks) at 10° C and 20° C mean heat coma temperatures in salinities ranging from 7.75‰ to 31.00‰ were 34.86° C and 40.26° C respectively, while in FW they dropped to 32.20° C and 36.21° C respectively. Upper lethal limits for both stocks were 44.5° C (at 31.00‰).

Measured by oxygen-electrodes at five-degree intervals from 5° C through 50° C, aerial oxygen uptake rates in 10° C-acclimated snails had a Q_{10} of 2.13 (5° C–35° C) and declined sharply at higher temperatures. In 20° C-acclimated stocks a Q_{10} of 2.19 (5° C–35° C) was followed by a relatively constant rate of oxygen uptake from 30° C to 40° C ($Q_{10} = 1.10$). There was an indication of what has been termed a "reverse" pattern of acclimation in the respiratory response to temperature in 10° C and 20° C stocks.

Oxygen uptake rates (5° C–50° C) in SW (seawater; 31.00‰) as percentages of those in air averaged only 8% (20° C-acclimated) to 20% (10° C-acclimated). In 20° C-acclimated snails, rates at 15.50‰ and 31.00‰ SW were twice the rates in FW. In 10° C-acclimated stocks rates did not differ between SW and FW. The indication of "reverse" acclimation persisted in higher salinities. Both stocks at 20° C had "oxygen-dependent" uptake patterns over a series of reduced oxygen tensions.

As with uptake rates, the Q_{10}s for water (1.23–1.74 at 10° C; 1.47–1.95 at 20° C) are lower than those for air, and in higher salinities there is a similar zone of respiratory thermal regulation (30° C–45° C) in 20° C-acclimated stocks. In terms of its respiratory physiology, *Melampus* is clearly an air-breathing snail colonizing the high littoral environment.

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Variation in the ontogenetic development of radial ornamentation in pelecypods and brachiopods. STEVEN W. MITCHELL.

Considerable variation has been found to occur in the ontogenetic development of radial ornament in selected species of living and fossil brachiopods and pelecypods. At a given growth stage, radial ornament develops more rapidly (i.e., the population is maturing faster) in environments progressively nearer the equator. Recent species of living brachiopods (*Hemithiris psittacea, Notosaria nigricans, Terebratulina retusa, Terebratulina septentrionalis, Terebratulina unguicula*) and pelecypods (*Anadara ovalis, Anadara transversa, Argopecten gibbus, Argopecten irradians, Codakia orbicularis, Donax variabilis, Noetia ponderosa*) demonstrate this trend convincingly. At a given growth stage of these species, populations progressively nearer the equator exhibit an increase in the development of radial ornament. This trend seems to exist in a wide variety of other living and fossil brachiopods and pelecypods.

Such variation in the ontogenetic development of radial ornament is attributable to two possible factors: 1) photoperiodism, and 2) environmental temperature. While photoperiodism may be significant, its effect on ontogenetic development of radial ornament in pelecypods and brachiopods has not been investigated. Comparison of rates of increase of radial ornament with temperature (using the average daily summer temperature or the average daily temperature for the entire year) indicates that the relationship between the two is nearly linear. It is known that environmental temperature controls the ontogenetic development, growth rate, and longevity of poikilotherms. Generally, a warmer environment will lead to faster ontogenetic development and higher growth rates. Colder environments will tend to slow growth and produce longer-lived adults. The observed variation in the development of radial ornament in pelecypods and brachiopods appears to be the result of temperature control of ontogenetic development and growth.

If such a relationship between temperature and ontogenetic growth can be assumed to have existed during the Phanerozoic, then relative differences in average bottom temperatures should be preserved as variations in the development of radial ornament in brachiopods and pelecypods. Phanerozoic paleotemperature gradients derived by this technique appear reasonable and agree with paleogeographic reconstructions based on continental drift.

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Effects of pentobarbital on the squid giant synapse. KATHLEEN MORGAN.

The use of the squid giant synapse allows a more detailed study of the effects of barbiturates than in other model systems because of the larger size of the presynaptic terminal. I recorded intracellular potential pre- and postsynaptically at 17–18° C. I hyperpolarized the postsynaptic terminal for the measurement of excitatory postsynaptic potentials (EPSPs). It took 2 hours to reach steady state levels in the drug effects. At pH 6.5, pentobarbital at 0.3 to 1.0 mM decreased the EPSP height in a reversible, dose-related manner. At pH 8.0 the same concentrations were significantly less effective. All other measurements were at pH 6.5. There was no change pre- or postsynaptically in action potential height or resting potential.

In the presence of 10^{-7} g/ml TTX and 5 mM 4-aminopyridine to block sodium and potassium currents, a calcium dependent spike associated with transmitter release can be recorded from the presynaptic terminal. Solutions containing 40 mM calcium allow the "calcium spike" to be recorded more easily and were used in this part of the study. 0.3 to 1.0 mM pentobarbital reversibly reduced the duration of the calcium spike from 16% to 86%, respectively. The height of the plateau was reduced from 5% to 36%, but was less often reversible. The decrease in the calcium spike and the EPSP occur at the same concentrations but because of the large size of the calcium spike in high calcium solutions and the relatively short time course of the EPSP, the relationship between the two effects was not determined. The synapse was more subject to fatigue in the presence of pentobarbital in a dose-related, reversible manner at concentrations as low as 0.1 mM where there was no measurable change in EPSP height.

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Electrophysiological determination of the function of the lagena in terrestrial amphibians. PETER M. NARINS.

The lagena is a cup-like otolithic endorgan found in the labyrinth of fishes, amphibians, reptiles and birds; it is absent in mammals. Lowenstein and Roberts (1950, *J. Physiol.,* **110**: 392) recorded from single lagenar fibers in the isolated labyrinth of the thornback ray (*Raja clavata*) and found that their spontaneous firing rates were maximum when the animal was in a normal, upright position; firing rates decreased markedly when the preparation was subjected to either lateral or fore-and-aft tilting. Furthermore, they reported that the lagena in the thornback ray was not vibration sensitive.

Glass micropipettes filled with 3 M KCl (impedance 20–60 meg) were used to record extracellular activity from single fibers in the lagenar branchlet of adult leopard frogs (*Rana pipiens*). The branchlet was exposed surgically using a ventral approach. The animal was then inverted and placed on a tiltable table in a normal, prone position. Next, the electrode was inserted in the lagenar branchlet from beneath, through a hole in the table, while viewing with a dissecting microscope and mirror arrangement.

Both spontaneously active and silent single fibers were isolated. Spontaneous rates ranged from 20–40/sec. These fibers ceased firing when the animal was tilted laterally $\leq 3°$ from the horizontal in either direction. The non-spontaneous fibers responded to substrate-coupled vibratory stimuli within a narrow range of lateral tilt ($\pm 3°$). Unilateral loss of the lagena in *R. pipiens* resulted in a slight ipsilateral head tilt, whereas a frog with bilateral loss of the lageneae showed only an oscillation of the head following movement. These preliminary results suggest that the lagena in the frog serves as a fine adjustment indicator when sensing the horizontal spatial orientation.

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α-Bungarotoxin blockade of sperm cell function. LEONARD NELSON.

Membrane-seated cholinergic processes appear to be involved in regulation of sperm cell motility. Our experiments have shown that a number of cholinomimetic, anticholinesteratic and acetylcholine-receptor blocking agents, as well as drugs known to inhibit choline acetyltransferase, cause dose-dependent increase and decrease in sperm swimming speed. Sea urchin sperm cells respond particularly sensitively to nicotine, from complete and partial inhibition over the concentration range from 10^{-3} to 10^{-7} M, with almost a doubling of swim rate at 10^{-9} M. Substantiation of the nicotinic nature of the acetylcholine receptor (and of the initial postulate) was obtained upon treatment of *Arbacia* sperm suspensions to α-bungarotoxin. The toxin is a component of the venom of the banded krait, *Bungarus multicinctus,* which contains several neuromuscular blocking agents. The purified α-bungarotoxin, an 8000 mol wt polypeptide, binds acetylcholine receptors with a very high degree of specificity (the receptors have been shown by other investigators using electrophysiological methods to be located on the outer surface of muscle membranes). Nanomolar amounts of the toxin very quickly reduced the relative number of swimming sperm cells on a microscope slide to about one tenth of the controls, with fewer than 1% showing only ineffectual flagellar twitching at micromolar concentrations. Quantitative determination of motility changes by the sedimentation-orientation method showed a 50% reduction in the swimming speed after less than 5 minutes in micromolar toxin, while after 10 minutes even 10 nanomolar bungarotoxin caused a 25% reduction in speed. From the flagellar dimensions, assuming a toxin binding site density comparable to that of rat diaphragm post-synaptic membranes (10,000 sites/μm^2), calculations suggest that the binding sites may be uniformly distributed on the total surface of the flagellum. This is based on the observed 50% or greater reduction in motility in micromolar bungarotoxin. Electron microscopic autoradiography of spermatozoa treated with radioiodine labeled α-bungarotoxin will be employed to establish whether the distribution is uniform or differential.

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Distribution and multiple activities of liver elongation factor 1 in temperature acclimated toadfish. JENNIFER NELSON AND AUDREY HASCHEMEYER.

Previous studies in this laboratory have indicated that levels of polypeptide elongation factor 1 (EF–1) in toadfish liver supernatants correlate with actual rates of polypeptide chain assembly measured *in vivo*. The increase in activity associated with cold acclimation in both summer and winter fish has been shown to be almost entirely associated with the lower molecular weight forms (50,000–200,000 daltons) of EF–1. Further purification of the small form by CM-Sephadex and Sepharose-4B chromatography has led to the separation of two species with ^{3}H-phe-tRNA binding activity at about 70-fold purification over post-ribosomal supernatant. The ratio of binding to polymerization activity is 1.9 for one form, similar to that of post-ribosomal supernatant, and 3.5 for the other. Kinetic experiments show stoichiometric binding only for the latter, whereas catalytic activity is indicated in the former and in unfractionated EF-1. The results suggest the existence of multiple activities in toadfish liver EF-1 analogous to the EF-Tu and EF-Ts factors of bacterial systems.

The distribution of toadfish liver EF-1 binding activity is about 88% in 100,000 × g supernatant and 12% in the pellet, primarily in the ribosomal overlay. About 2% of total activity is recovered in ribosomes purified by deoxycholate treatment and sedimentation through 2 M sucrose. Stimulatory activity toward binding by the purified binding component of EF-1 described above is found in the 0.5 M NH_4Cl wash of ribosomes, proportional to the endogenous binding activity of the ribosomes preparation. The distribution of the two activities does not appear to differ for warm and cold acclimated fish.

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Identification of an intermediate in the metabolic conversion of proglucagon to glucagon. BRYAN D. NOE AND G. ERIC BAUER.

Following a preincubation in the absence of isotopes, islet tissue from angler-fish was pulse incubated for 20 minutes in the presence of ^{3}H-tryptophan and ^{14}C-isoleucine. Tissue was removed for extraction at the end of the pulse period and at various times during chase incubations in the absence of isotopes. Radioactive proteins in extracts were analyzed by gel filtration and polyacrylamide gel electrophoresis (PAGE). Radioimmunoassay (RIA) was used to determine the distribution of immunoreactive glucagon. Proinsulin was the only molecule labeled with ^{14}C-isoleucine following the 20-minute pulse. Two glucagon immunoreactive molecules having approximate molecular weights of 11,400 (proglucagon) and 9000 became labeled with ^{3}H-tryptophan during the pulse period. With increasing duration of chase incubation, ^{3}H-tryptophan labeling appeared and increased in a molecule possessing glucagon immunoreactivity and a molecular size similar to glucagon. Simultaneously, ^{3}H-radioactivity in the 11,400 mol wt molecule continuously declined whereas ^{3}H-label in the 9000 mol wt molecule initially increased and subsequently decreased. This latter pattern is consistent with the pattern of labeling which might be expected for a conversion intermediate. Confirmation of the presence of glucagon immunoreactivity in the large ^{3}H-tryptophan labeled molecules was obtained by RIA of gel slice extracts following low pH PAGE. Assessment of the distribution of radioactivity in polyacrylamide gels following high pH PAGE of the proteins recovered from the insulin and glucagon containing portions of the gel filtration eluates proved that ^{3}H-tryptophan became incorporated into glucagon exclusively, and ^{14}C-isoluecine was incorporated only into insulin. These results provide evidence for the presence of a 9000 mol wt intermediate in the metabolic conversion of angler-fish proglucagon to glucagon.

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Spicule formation by micromeres isolated from embryos of Arbacia punctulata. KAYO OKAZAKI.

It has recently been reported that micromeres isolated from embryos of three species of Japanese sea urchins continue on the same developmental course as those in the normal embryo and finally form spicules much like those in normal plutei (Okazaki, 1975, *Amer. Zool.,* **15**:

567). The experiment was repeated using the American sea urchin, *Arbacia punctulata.* Essentially the same results were obtained, but the methods of isolating the micromeres required some modification.

Eggs were fertilized in a test tube with a small amount of sea water. Thirty seconds after insemination, seven times as much calcium- and magnesium-free sea water (CMF) as the volume of the egg suspension was added. After two minutes, the medium was decreased to 2–3 ml and fertilization membranes were removed by pipetting. The demembranated eggs were kept in sea water until the 16-cell stage, when they were collected and washed three times with CMF and resuspended in calcium-free sea water (CF). Blastomeres were dissociated by gentle aspiration through a pipette. The micromeres were isolated from the other blastomeres by using a sucrose density gradient after Spiegel and Spiegel (1975, *Amer. Zool.,* **15**: 583), although CF was used instead of CMF. One to two thousand of the isolated micromeres were transferred to a 100 × 15 mm Falcon plastic dish containing 20 ml of culture medium. The culture media were millipore filtered sea water containing 2% of horse, fetal calf, new born calf or calf serum (Grand Island Biological Co.). The four kinds of sera gave essentially the same experimental results. More than 60% of the cell aggregates composed of descendants of isolated micromeres formed spicules *in vitro* by the time the control larvae reached early pluteus stage. About half of the spicules showed the complex structure characteristic of this species. Others were simple in structure.

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Island biogeographic theory as a major principle for understanding marine epifaunal communities. RICHARD W. OSMAN.

The study of the fauna and flora of islands has contributed greatly to ecologic and evolutionary theory. Long noted on islands has been the log-log linear relationship between the area of an island and the number of species present on it. The species equilibrium theory proposed that this relationship resulted from a balance between immigration of species onto an island and extinction of species already present. The increasing number of species with increasing area was predicted to result from a decreased probability of extinction. Immigration was predicted to be an inverse function of the distance from the source of immigrants. To the marine epifaunal invertebrates, their rock substrates are also distributed as islands. The colonization of both rocks and experimental slate plates was studied on Nonamesset Island for a period of 27 months in order to test the equilibrium model. It was found that the colonization of the plates resulted in an equilibrium number of species in which extinction and immigration were balanced. The equilibrium number of species was found to be a function of substrate size. However, unlike the model this resulted from changes in the immigration rate. Marine epifauna also reproduce and colonize rocks seasonally, and this produces a changing immigration rate. These fluctuations in immigration caused extreme variation in the shape of the colonization curves of plates initially immersed in different months and also produced a seasonal cycle in the number of species present at equilibrium. Finally, it was found that physical disturbance affected the number of species on a substrate. Intermediate amounts of disturbance yielded the highest diversities. Thus, the model was sufficiently robust to incorporate many factors.

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Effects of hornet venom on squid axon membranes. J. L. PARMENTIER AND T. NARAHASHI.

Venom from the social wasp *Vespula arenaria* was collected locally by inducing the insects to sting an electrically-charged wire grid which covered a sheet of parafilm. The venom was dissolved in artificial sea water as varying concentrations of wasp droplets (Vespentropfen) which resulted from each individual sting. The crude venom solutions were added directly to a chamber which held a squid giant axon under voltage clamp conditions. All experiments were at room temperature.

Vespula toxin was found to have effects similar to those of local anesthetics in that both the sodium and potassium conductances were reversibly suppressed in a manner dependent upon the concentration (1–7 vt/ml). The time to peak current was not greatly affected, suggesting that the membrane sodium kinetics are not altered, and that these effects may operate directly on the conductance channels. Leakage current was increased and conductance irreversibly suppressed only when the concentration was raised to greater than 10 vt/ml. This effect of high concentrations is probably due to lytic, non-neurotoxic components of the venom. It is possible that specific pharmacologically active agents in the *Vespula* toxin may be responsible for the effects reported here, but more detailed work must await fractionation of the venom into its active consituents. The ease with which large quantities of insect venom may be collected using the charged grid system should allow its biochemical analysis by standard laboratory techniques. This raises the possibility of producing medically significant antisera to hornet and other aculeate venoms through standard immunological processes.

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Calcium control of a cyanide insensitive respiration in Arbacia punctulata *eggs, and its relation to echinochrome.* George Perry and David Epel.

Soon after fertilization oxygen consumption of *Arbacia punctulata* eggs increases 5–10 fold. About 30% of the fertilized egg respiration is insensitive to 5 mM KCN. However, more than half of the O_2 consumption is CN-insensitive during activation with the calcium ionophore A23187. Yet respiration of mitochondrial preparations are completely inhibited by 1 mM KCN. Addition of A23187 to fertilized eggs, already inhibited by KCN, still causes a sudden increase in O_2 consumption.

Calcium addition to egg homogenates induces a large, cyanide-insensitive oxygen consumption, which is EGTA reversible. This Ca^{+2}-activated respiratory activity sediments at $500 \times g$ with the pigment granule fraction, although the cytochrome-c-oxidase activity remains in the supernatant. Triton X-100 (0.1%) solubilizes the calcium-induced O_2 consumption along with the previously particle-associated echinochrome. This napthoquinone was extracted from egg homogenates with acidified ethyl ether, dried, and dissolved in ethanol. The preparation consumed oxygen in response to calcium addition, along with an O_2-independent immediate color change and subsequent precipitation. The preparation retains activity after heating. Activity co-purifies with napthoquinone during gel filtration, crystallization (crystals provided by Dr. Eric Ball), and cellulose thin layer chromatography.

Strontium and barium, but not magnesium, will substitute for calcium. The activity is concentration dependent, showing saturation at high Ca^{++} levels. Reaction does not occur below pH 6 and increases with greater alkalinity. Since free Ca^{+2} increases at fertilization, echinochrome-related respiration can account for the cyanide-insensitive O_2-consumption of *Arbacia* embryos. The role of this napthoquinone-related respiration is unclear, but its redox potential is such that it could accept electrons at physiological pH.

Intracellular recordings from photoreceptors of the squid retina. L. H. Pinto and J. E. Brown.

Intracellular recordings were made from rhabdomeric segments of *Loligo* photoreceptors under infra-red observation. Resting membrane potential was about −65 mV. The waveform of the receptor potential in response to a dim stimulus consisted of monotonic depolarization at on and rehyperpolarization at off. In response to bright stimuli there was a transient depolarization that overshot OV, then declined to a plateau. For some cells there was an after hyperpolarization. In solutions with 98% of the Na replacd by Li or choline (low Na SW) responses reversibly disappeared in about 1–2 min and did not recover for as long as 5 min. During exposure to low Na SW polarization of the membrane with + 1 μA of extrinsic current did not recover any receptor potentials. Thus, the bulk of the light-induced current is carried by Na ions. However, in cells, previously injected with EGTA, exposure to low Na SW resulted only in response diminution. Exposure to (Ca)o of 1 mM or injections of EGTA into the cells caused the responses to become square and large. This is consistent with the interpretation that the transition from the peak of the transient-depolarization to plateau deplorization is causually related to increased free $(Ca)_{in}$. The retina is very sensitive to hypoxia, and in order to obtain stable recordings we had to maintain $PO_2 = 740$ mm Hg and exchange the contents of the perfusion dish every 3 sec.

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Ionic regulation of transmitter uptake in squid brain synaptosomes. HARVEY B. POLLARD AND JEFFERY L. BARKER.

Uptake of transmitter into the presynaptic terminal is thought to be an important mechanism for termination of synaptic transmission. It is possible that the ionic microenvironment of the nerve ending may modulate the uptake of different transmitters in specific fashions. To test this hypothesis, we studied the effects of H^+, Na^+, K^+, Ca^{++} and Mg^{++} on the uptake of eight different transmitters by synaptosomes derived from squid brain. The uptake of L-noradrenaline, dopamine, serotonin, octopamine, glutamate, GABA, choline and glycine were examined over the pH range of 6.0–9.5. The pH profiles of each substrate were different, with optimal values distributed over the pH range. The titration curves bore no relation to the pK_a values for the substrates, leading us to conclude that pH dependences were probably related to conformational changes of enzymes in the synaptosomal membrane involved in permeation. The omission of Mg^{++} from the medium had little effect on uptake, while omission of Ca^{++} resulted in a small but significant non-competitive activation of most uptake systems. Under conditions of zero Na^+, all systems but glycine were substantially depressed. GABA was unique in that it had a sigmoidal dependence on (Na^+), with a $k_{\frac{1}{2}} = 300$ mM. All K^+ curves were maximal in the physiological range (10 mM K^+), and were greatly inhibited at both zero K^+ and 100 mM K^+. The zero K^+ effect was independent of calcium, while the inhibition at 100 mM K^+ was abolished in a Ca^{++}—free medium. We conclude that changes in the ionic composition of the synaptic micro-environment may play an important role in the differential modulation of synaptic transmission. The mechanisms of these specific effects may involve action on transport enzymes in the synaptosomal membrane.

Behavioral and physiological aspects of water relations in the high littoral snail, Melampus bidentatus *Say.* CHRISTOPHER H. PRICE AND W. D. RUSSELL-HUNTER.

Melampus bidentatus, the primitive, amphibious pulmonate snail of high littoral areas of salt marshes, is subjected to a wide range of water conditions, including extremely dry during neap tidal cycles in summer. *Melampus*, with no operculum, nor capacity to burrow or to form a true epiphragm, must cope with desiccating conditions by a combination of behavioral and physiological adaptations. In the laboratory, rates of body water (W_b) loss in adult *Melampus* are high [e.g., 6.2% W_b/hour, at 50% relative humidity (rh) and 20° C], and inversely proportional to both relative humidity and animal size. Individual rates of water loss are reduced in aggregated groups when compared with rates from an equal number of snails kept separated during the drying process. Tolerance of desiccation is remarkable, with most animals surviving W_b losses of 75–80%. Resorption of water is rapid—from a level of 50% W_b, 90% of the loss can be regained 40 min after providing free water. The significance of some aspects of these studies to field conditions was assessed by collecting animals this summer on hot, dry days during neap cycles. Field weights (and subsequent weights on rehydration) could be compared with corresponding expected values from a regression of normal, hydrated weight on shell length, and then expressed as percentages of the expected. Such mean weight disparity (MWD) percentages were often near -10% in field groups thought to be water stressed (e.g., July 30, 34° C, 52% rh, areas exposed for 24 hours: $N = 46$, $MWD = -8.28\%$, ranging to -22.6%; rehydrated $MWD = -2.3\%$; t-test significant at 0.001). Under drying field conditions, animals are inactive and positively geotrophic: individuals rest under rocks or dirt clumps, and bury under loose soil or dead grasses; and populations often form aggregated groups on the marsh surface. Such behavioral patterns maximize chances of locating less-harsh microclimates. Coupled with the physiological insurance of extreme desiccation tolerance and rapid rehydration, these patterns ensure the "success" of *Melampus* in this marginal and often severe habitat.

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Laying of egg capsules in Busycotypus *induced by nervous system extracts.* JEFFREY L. RAM.

Busycotypus (= *Busycon*) lays it eggs in strings of disciform egg capsules which it produces over the course of several days. The two species found in Woods Hole, Massachusetts, *B. canaliculatum* and *B. carica,* produce egg cases which are distinctly different in shape. The present study indicates a possible role of neurohormones in egg capsule production. All experiments were done during the summer egg-laying season (July and August, 1975).

Nervous systems of large (250–700 g, with shell) female *B. canaliculatum* were homogenized in 0.2 M sodium phosphate buffer (pH 7.2) or in filtered sea water and centrifuged at 350–500 × g for 2 min. The supernatant was injected into the pedal sinus of large female recipients. 13 out of 33 first time recipients produced egg capsules. Animals which had produced egg capsules at least once reliably produced egg capsules upon subsequent injections (19 out of 22 trials). Except where otherwise stated, all experiments below used only reliably laying recipients. It should be noted that the capsules produced by extract injection never contained any eggs.

One 0.5 ml injection of one quarter of a nervous system was sufficient to cause one egg case to be produced on most occasions. Neither phosphate buffer nor filtered sea water injections caused capsule production (0/10 and 0/6 trials, respectively).

B. canaliculatum extracts produced egg cases in *B. carica* (2 out of 4 first time recipients). *B. carica* caused capsule laying in 3 out of 3 *B. canaliculatum* recipients. Capsule shape was specific to the recipient. Nervous system extracts of male *B. canaliculatum* (200 to 240 g) caused egg capsule laying (3 out of 6 extracts), as did extracts from comparably sized females (5/6). The egg capsule inducing substance was found in parietal ganglia (7/7), less reliably in cerebral-pleural ganglia (3/5), and never in pedal (0/5), buccal (0/5), or visceral (0/6) ganglia or in esophagus (0/2).

Supported by an MBL junior investigator scholarship.

Improved photosensitive devices for observing in vivo *bioluminescence spectra.* GEO. T. REYNOLDS.

Two separate photosensivtive system have been assembled: one extending the spectral range for observing bioluminescence by means of photomultipliers; the other an improved image-intensification-spectroscope system for observing *in vivo* spectra.

An RCA 8852 ("Quanticon") photomultiplier with an extended red cathode response is cooled to −30° C, and fitted with a magnet to restrict sensitivity to a small portion of the cathode. The dark current count rates are a few per second. Assembled with a Corning CS2–61 filter the photomultiplier provides detection efficiency of $\gtrsim 5\%$ between 6100Å and 8700 Å ($>1\%$ from 6000 Å to 9000 Å) with very low detection efficiency either side of this band. The system thus permits a search for bioradiation beyond the long wavelength limit of the dark adapted eye (scotopic vision is limited to wavelengths <6400 Å). With this system used in the current mode, no radiation above noise was observed from the dinoflagellates *Gyrodinium cohnii* and *Glenodinium foliaceum,* which also are not observed to luminesce in the range of human eye sensitivity. A systematic search among other potentially radiating species is underway.

The image intensified spectroscope previously reported has been improved by the addition of a photomultiplier set to detect the image intensifier output by means of a light pipe so that spectra of optimum exposure are obtained, even from intermittant weak flashes which must be integrated to provide a usable record. These records are measured by means of a digitized densitometer computer corrected for overall system nonuniformities and printed out at 4 Å intervals. Preliminary observations with this improved system have been made on *Pyrocystis lunula, Gonyaulax polyedra,* and aequorin.

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Increase in ciliary length of Arbacia *blastulae by Concanavalin-A or trypsin treatment.* M. A. Riederer-Henderson and Joel L. Rosenbaum.

A number of substances when applied to either unfertilized or fertilized sea urchin eggs cause animalization—a process in which development is inhibited at the blastula stage and which is characterized by an increased number of long cilia on the blastula. Here we report that normal, hatched blastulae (which have primarily short, 25 μ cilia except for a few long, 30–65 μ cilia at the apical tuft) can be induced to form long cilia around most of their circumference by treatment with trypsin (0.008–0.10%). Other animalizing agents, *e.g.* zinc, sodium thiocyanate, Evans blue, thioctic acid do not induce formation of long cilia when they are applied to blastulae. Long cilia first appear around the apical tuft after 7 hr in trypsin, and by 22 hr (21° C) most of the blastula is covered with the long cilia. Length distribution studies on cilia isolated at different times indicate that the percentage of long cilia increases from 10% in the normal blastula to 60% in the 22 hr trypsin-treated embryo. The trypsin is not effective in the presence of soybean trypsin inhibitor. Only the blastula and gastrula stages can be induced to form long cilia; the prism and pluteus cannot. Once development is inhibited and the first long cilia appear, the trypsin effect cannot be reversed. Indeed, if the blastulae with long cilia are removed from trypsin for 10 hr, the cilia remain long: if the cilia are detached, the blastulae regenerate long cilia. The long cilia move poorly as do the long cilia on normal blastulae. Concanavalin-A (0.05–0.10 mg/ml) is also effective in causing blastulae to develop long cilia, but only over $\frac{1}{4}$ to $\frac{1}{2}$ of the surface of the blastula. The trypsin treatment appears to be releasing the short cilia from their normal length control so that ciliary precursors can continue to assemble. Ultrastructural and biochemical studies are in progress to determine how trypsin is functioning in this process.

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Extrinsic birefringence and dichroism changes in squid giant axons. W. N. Ross, L. B. Cohen, B. M. Salzberg, N. Kohn, and A. Grinvald.

The absorption of light by dyes bound to giant axons from the squid, *Loligo pealii,* changes when membrane potential is altered. In experiments where the direction of light propagation is perpendicular to the longitudinal axis of the axon, the absorption changes for several dyes were found to depend upon the plane of polarization of the incident light. The absorption change of a merocyanine dye (dye I, 1974, *J. Membr. Biol.*, **19**: 1) was larger with light polarized perpendicular to the longitudinal axis than with light polarized parallel to the axon at wavelengths between 550 and 600 nm. At wavelengths between 450 and 550 nm and between 600 and 630 nm, the converse was found. When the absorption change for light polarized parallel to the axon was subtracted from the change for light polarized perpendicularly, an approximately bell-shaped curve with a peak at 550 nm was obtained. As expected from the existence of dichroism in the absorption change, there was also a dye dependent birefringence change that could be measured when the axon was placed between perpendicular polarizers, with the incident light polarized at 45° to the long axis of the axon. For depolarizations there was a net decrease in optical retardation at wavelengths from 550 nm to the longest wavelengths tried (700 nm), and an increase at wavelengths shorter than 550 nm. The general shape of the spectrum of the biregringence change was in agreement with the prediction from a Kramers-Kronig transformation of the dichroism spectrum. With a merocyanine-rhodanine dye, 5-[(1-γ-sodium sulfopropyl-4(1H)-quinolylidene)-2-butenylidene]-3-ethylrhodanine, both the dichroism and birefringence changes were larger and easily detected in a single sweep. The size of the birefringence change for both dyes was directly proportional to the change in membrane potential. These birefringence signals may prove to be useful monitors of membrane potential because wavelengths outside the absorption band can be used, avoiding any photodynamic damage to the preparation or bleaching of the dye.

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New and more sensitive molecular probes of membrane potential: simultaneous optical recordings from several cells in the central nervous system of the leech. B. M. Salzberg, L. B. Cohen, W. N. Ross, A. S. Waggoner, and C. H. Wang.

We have extended our search for sensitive molecular probes of membrane potential to include more than 600 dyes. Among these we have recently found four molecules which provide fluorescence or absorption changes which vary with membrane potential and can be measured with signal-to-noise ratios substantially larger than those obtained (∼5:1) with the potential sensitive probe Merocyanine 540 (dye I, 1974, *J. Membr. Biol.*, 19: 1). Measurements were made on a 4 mm length of voltage clamped giant axon from the squid, *Loligo pealii.* Two of the dyes were most sensitive in the absorption mode, and two in the fluorescence mode. They included (with single sweep signal-to-noise ratio and the mode in which it was obtained in parentheses) a merocyanine, 5-[(1-γ-propyltriethylammonium bromide-4(1H)-pyridylidene)-2,4-hexadienylidene]-1,3-diethyl-2-thiobaribituric acid (10:1, fluor.); two dyes of the oxonal class, Bis-[1,3-dibultyl-barbituric acid-(5)]-pentamethinoxonol (17: 1, fluor.) and Bis-(3-carbopropoxy-1-phenyl-2-pyrazolin-5-one)-pentamethinoxonol (10:1, abs.), and, the most sensitive, a merocyanine-rhodanine dye, 5-[(1-γ-sodium sulfopropyl-4-(1H)-quinolylidene)-2-butenylidene]-3-ethylrhodanine (50:1, abs.). All four of these new probes inflict less photodynamic damage on nerve membrane than does Merocyanine 540, the merocyanine-rhodanine being 100 times less toxic in the presence of intense light and oxygen.

The merocyanine-rhodanine dye has been used to record simultaneously from three sensory neurons in the central nervous system of the medicinal leech, *Hirudo medicinalis.* Light guides, 1.5 mm in diameter, optically coupled to small silicon photodiodes were positioned, in the objective image plane of a microscope, over the images of three cells in the ganglion. One of the cells was impaled with a microelectrode, and stimulated past threshold. Absorption at 750 ± 50 nm was monitored in all three channels at once and only the channel corresponding to the stimulated neuron recorded an optical spike. We expect that this technique can be employed in an expanded version of this same apparatus, where 10–30 neurons are monitored simultaneously.

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Laser light scattering from a neurosecretory tissue. David B. Sattelle, David J. Green, and Kenneth H. Langley.

Laser light scattering spectroscopy was used to investigate particle motion in the pericardial organs of the lobster *Homarus americanus.* These organs contain numerous peripherally arranged neurosecretory cell processes.

Isolated pericardial organs were mounted under saline in a water-cooled (14–16° C) optical glass cuvette which was flushed with fresh lobster saline every six minutes. Light from a 5 mW He-Ne laser ($\lambda = 632.8$ nm) scattered from a 100 µm diameter circular area of the surface of the pericardial organ was detected by a photomultiplier and the intensity autocorrelation function obtained from a digital correlation computer. The autocorrelation function has a monotonically decaying time-dependent component which derives from moving scatterers and a time-independent component originating from stationary scattering structures.

One major change was noted in the correlation function when neurosecretory release was stimulated by increasing the potassium level in normal saline from 16 mM to 100 mM. During exposure to high potassium there was a reversible transient increase in the amplitude of the correlation function derived from moving scatterers relative to the part arising from stationary scatterers. In 11 out of 14 such responses from 8 pericardial organs, the amplitude increase appears to be related to events subsequent to discharge of neurosecretory material.

Our findings suggest that this technique can monitor particle motion in nervous tissues.

Significant age-dependent and locality-dependent changes occur in gene frequencies in the ribbed mussel Modiolus demissus *from a single salt marsh.* THOMAS J. M. SCHOPF, MARK D. OHMAN AND ROBERT BLEIWEISS.

Animals of approximately 3 and 9 years old were collected from near the mouth and the head of Little Sippewissett Marsh, Cape Cod, Massachusetts (1 km long, 300 m wide). Allele frequencies were scored in 48–62 animals in each age group from each locality utilizing polyacrylamide gel electrophoresis and histochemical staining for leucine amino peptidase (3 common alleles), phosphoglucose isomerase (3 common alleles), and tetrazolium oxidase (2 common alleles).

As animals age in the upper marsh, p (= frequency of slowest migrating allele) for LAP significantly decreases from 0.43 to 0.20, and q increases from 0.30 to 0.47; in contrast, q decreases from 0.41 to 0.21 as animals age in the lower marsh. Also, for 3 year olds, p is significantly lower in the lower marsh than the upper marsh; and for 9 year olds, q is significantly lower in the lower marsh than in the upper marsh. Significant differences also appear to exist in PGI allele frequencies. In contrast, for TO, no differences exist by age or by locality ($p = 0.30$). Thus some polymorphic loci for a given species change and others do not, therefore suggesting responses to different selective factors. As animals age, a significant *decrease* occurs in heterozygosity in LAP (but not in TO), in both the lower and upper marsh.

Physiologists may wish to consider genetic differentiation in interpreting responses of *Modiolus* of different ages or localities. And evolutionists may note that the environmental gradient in a marsh may result in as much genetic differentiation as is observed in species occurring over much greater distances with a lower gradient of environmental change.

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Ultrastructural localization of calcium in the early stages of fertilization in Arbacia *eggs.* HERBERT SCHUEL, CONSTANCE CARDASIS, LAWRENCE HERMAN, AND WALTER L. WILSON.

The discharge of the cortical granules by exocytosis and the detachment of the vitelline layer to form the fertilization membrane are normally completed in *Arbacia* eggs within one minute post insemination. Eggs were fixed in glutaraldehyde at intervals of 5, 15, 30, 45, and 60 sec post insemination, and post-fixed in osmium-antimonate mixture to demonstrate cation localization. There was a marked reduction in precipitate within eggs exposed to EGTA after fixation, suggesting that the deposits contained calcium. Preliminary X-ray microprobe analysis of some deposits confirmed the presence of calcium. There appeared to be a transient increase in deposits in the ground cytoplasm of the eggs at 5 sec post insemination. Large antimonate deposits were present in the lumen of discharging cortical granules and in the perivitelline space. The latter suggests calcium complexed to hyaline protein, a component of the secretory product. Deposits persisted on cortical granule membranes during exocytosis. Rod-containing vesicles exhibited precipitate in both unfertilized and fertilized eggs and were prominent in association with the Golgi apparatus 45 sec post insemination. Yolk platelets contained a fine homogeneous matrix precipitation, while a second more pale-staining structure resembling platelets contained deposits only along its outer membrane. A transient reduction in deposit on the plasma membrane was seen at 30 sec post insemination after detachment of the vitelline layer. Sperm cells at the egg surface had some deposit in the nucleus, and had heavy precipitation on the acrosome, mitochondria and tail.

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The effect of light intensity and temperature on the emergence of Cryptocotyle lingua *cercariae from the host,* Littorina littorea. KATHLEEN STRYESKI.

Cryptocotyle lingua (Creplin) is a digenetic trematode which undergoes larval development in the intermediate host, *Littorina littorea.* It has been found that the emergence of cercariae from the snail can be altered by changing environmental factors such as light intensity and water temperature. Infected gastropods partly submerged in water were exposed to intense light, moderate light, and darkness. These animals discharge the greatest number of cercariae under intense light, regardless of the water temperature. Emergence is significantly reduced in the absence of light, with moderate light intensity yielding intermediate numbers of cercariae.

The optimal temperature range for larval emission is from 21–25° C. Therefore, under ideal conditions (21–25° C, high light intensity) the maximal concentration of cercariae would be liberated. Larvae are also shed in considerable numbers when the host is exposed to water temperatures ranging from 16–20° C and 26–30° C. A greater number of cercariae is emitted in the lower temperature interval. Water temperature has a "threshold effect" in which cercarial release is altogether inhibited at specific low and high temperatures. For the parasite under discussion, low temperatures from 12–15° C and high temperatures from 36–38° C completely supress cercarial emergence.

At the optimal temperature and under constant illumination, the snails characteristically release the greatest number of cercariae from 10:00 AM through 1:00 PM daily. Complete darkness at this temperature tends to alter the time of greatest emergence in individual snails by several hours. The peaks of emission also become scattered as the snails are subjected to water temperature other than the optimal. The study of the emergence of cercariae can be utilized as an important diagnostic tool in the identification and control of larvae.

This study was performed in conjunction with the Marine Ecology Course at the Marine Biological Laboratory. I wish to thank Dr. Horace W. Stunkard for his advice and suggestions.

Studies on trematode parasites of birds: developmental stages of Odhneria *sp., (Microphallidae).* HORACE W. STUNKARD AND MICHAEL DISPEZIO.

Common prawns, *Palaemonetes vulgaris,* collected in Quissett harbor, sustain natural infections by encysted metacercariae. The parasities are clearly visible in living shrimps. Released from their cysts, the *Anlagen* of the reproductive organs and details of the excretory system were elucidated, and the worms were referred to the family Microphallidae. The flame-cell formula is $2\ [(2+2)+(2+2)]$. Cysts that had been removed from prawns, and living specimens of *P. vulgaris* bearing cysts, were fed June 27, 1975 to a recently hatched gull, *Larus argentatus,* and the bird was autopsied June 30, 1975. Approximately 60 cysts had been fed and 32 gravid worms were recovered from the intestine three days later. The worms are assigned to the genus *Odhneria* Travassos, 1921, whose type-species, *O. odhneri,* was found in the intestine of a yellow-crowned night-heron, *Nyctanassa violacea,* taken in Brazil. Other species referred to *Odhneria* include *O. raminellae* (Dery, 1958) Yamaguti, 1971, from the red-breasted merganser, *Mergus serrator,* taken at Ram Island, Barn Island and Clinton, Connecticut; *O. charadrii* (Cable, Connor and Balling, 1960) Deblock, 1972, from the plover, *Charadrius wilsoni,* taken at Cabo Rojo, Puerto Rico; and *O. limnodromi* (Schell, 1967) Yamaguti, 1971, from the dowitcher, *Limnodromus griseus,* taken in Idaho. The specific identity of the specimens from the feeding experiment is yet equivocal. It is reasonably certain that the gull is not the natural host and that the worms would have been expelled in a few days. But very young birds lack resistance to infection and parasites of diverse species may mature in them. The normal hosts are probably shore-birds, plovers, *etc.,* and these migrant species accumulate and disseminate their parasites throughout their ranges. The molluscan host has not yet been identified.

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Histone acetylation during the early stages of sea urchin (Arbacia punctulata) *development.* BARBARA A. TAYLOR AND CAROLYN J. BURDICK.

A correlation has been found between rate of histone acetylation and gene activation at the time of gastrulation in the sea urchin, *Arbacia punctulata.* Between the blastula and gastrula stages, there is a 2.5-fold increase in the rate of acetylation of a histone fraction consisting of slightly lysine-rich (F_{2a2} and F_{2b}) and arginine-rich (F_{2a1} and F_3) components, with a marked decrease in the fully differentiated pluteus stage. The acetylation of lysine-rich histone (F_1), on the other hand, is minimal and does not change significantly during this developmental period. This pre-gastrular increase in slightly lysine-rich and arginine-rich histone acetylation is not correlated with histone synthesis which was found to decrease during this same period. It is also not correlated with 1) increase in acetate uptake into the embryo; 2) decrease in the rate of histone deacetylation rather than increase in histone acetylation; 3) changes in acetyl coenzyme A pool size (the immediate precursor for histone acetylation) since none of these factors change significantly during the period studied. The results thus suggest that increased histone acetylation may be at least one preparative factor for the activation of new genes at gastrulation.

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Detection of free calcium ions in amoebae by aequorin luminescence. D. L. Taylor, G. T. Reynolds and R. D. Allen.

Purified aequorin dissolved in a stabilization solution (Taylor *et al.*, 1973, *J. Cell Biol.*, **59**: 378) was microinjected into the giant amoeba *Chaos carolinensis* to a final concentration of *ca.* 10^{-4} mg/μl. The injected cells were viewed with an image intensifier with a gain of *ca.* 7.0×10^5 (Reynolds, 1972, *Quart. Rev. Biophys.*, **5**: 295). The limit of calcium detection with the above aequorin concentration was *ca.* 3.5×10^{-8} M free calcium in a $Ca^{++} - EGTA$ buffer. Upon recovering from microinjection (Taylor, 1974, *Biol. Bull.*, **147**: 501) the cells failed to exhibit spontaneous luminescence. Luminescence was induced, in parallel with cellular movements, with sufficiently strong electrical or mechanical stimuli. However, weaker stimulii could induce cellular movements without causing luminescence. A single electrical stimulus of *ca.* 5.0 to 10.0 volts/cm for 50.0 msec caused pseudopods to orient from the anode towards the cathode. Further stimulation produced luminescence on the anodal side of the amoeba with the continuation of streaming towards the cathode. In addition, weaker luminescence was sometimes observed simultaneously at the advancing cathodal end. Upon stimulation, cells luminesced for *ca.* 5 sec, and repeated stimulation caused cells to become monopodial as observed in cells microinjected with a contraction solution (Taylor, 1974).

The present results indicate that contractions are induced by calcium at the site of entry into the plasmalemma after electrical stimulation. Since the physiological calcium threshold for contraction is *ca.* 7.0×10^{-7} M, the failure to observe luminescence in unstimulated amoeba could be explained by: 1) the presence of natural calcium chelators and sequestering systems that effectively compete for free calcium ions and 2) the presence of magnesium ions or other substances that might compete with calcium for binding sites on aequorin.

Measurement of gas vacuole formation rates in a blue-green alga. B. A. Triplett and A. E. Walsby.

Gas vacuoles are made up of submicroscopic, hollow structures, termed gas vesicles, which provide planktonic blue-green algae with buoyancy. By varing the proportion of the cell volume occupied by gas vesicles, these organisms are able to regulate their buoyancy and select specific stations in the vertical water column.

The volume occupied by gas-vesicle space inside a dense suspension of algae can be calculated by the change in specific gravity of the suspension which takes place when the gas vesicles are collapsed by applying pressure. The volume of cells in the suspension can be obtained by subtracting the volume of interstial space. This we have determined by finding the volume of the suspension available to ^{14}C-labelled dextran (mol wt 16,000 daltons). Our measurements indicated that gas vacuoles occupy about 3.5% of the cell volume when the cells are neutrally buoyant, and can occupy more than 10% of the cell.

Measurements of cell gas-vacuolation made by turbidity measurements indicate that, in the blue-green alga, *Anabaena flos-aquae,* the lower degree of gas-vacuolation at high light intensities is a consequence of the increased incidence of collapse (see Grant and Walsby, 1975, *Biol. Bull.,* **149**: 428) rather than of decreased gas vesicle formation rates. In an attempt to follow formation in the absence of collapse, we have attempted to remove cell turgor pressure by placing the alga in an osmoticum of non-penetrating solutes such as mannitol and sucrose. Turgor pressure measurements made by the gas vacuole method indicate significant penetration of these solutes over several hours.

This work was supported by a grant, BMS-73-06800 from the National Science Foundation.

Calcium ionophore A23187 induced block to sperm penetration of Arbacia *eggs is prevented by protease inhibitors.* W. TROLL, H. SCHUEL, AND L. LORAND.

Arbacia eggs secrete a trypsin-like protease at fertilization or upon stimulation by calcium ionophore A23187 (Ca-I) which promotes the detachment of the vitelline layer to form the fertilization membrane. The latter acts as a mechanical barrier to prevent penetration of the egg by sperm. Protease activity (assayed fluorometrically using succinylated protamine as substrate) in the secretory product recovered from eggs 5 min following insemination or Ca-I activation was completely inhibited by soybean trypsin inhibitor (SBTI), antipain, and leupeptin. These protease inhibitors and also benzamidine were found to retard the elevation of the fertilization membrane from Ca-I activated eggs. As a result, subsequently added sperm were able to enter and fertilize Ca-I stimulated eggs, presumably at the numerous sites where the vitelline layer remained attached to the plasma membrane. Ca-I activated eggs developed only as far as the streak stage, even if the Ca-I was subsequently removed by extensive washing. However, Ca-I activated eggs fertilized in the presence of SBTI developed into normal larvae under similar conditions. These data suggest that the ionophore triggers the secretion of a protease from the eggs which promotes the block to polyspermy by raising the intra-cellular concentration of free calcium. Sperm may stimulate eggs by a similar mechanism during normal fertilization, as described by Schuel, Cardasis, Horman, and Wilson (1975, *Biol. Bull.,* **149**: 446). Furthermore, Ca-I appears to be incomplete parthenogenetic agent and may also be toxic to the eggs.

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Radiation-induced cleavage delay in Chaetopterus *eggs.* GOMATHY VISWANATHAN AND RONALD C. RUSTAD.

X-irradiation of either the eggs or the sperm of sea urchins is known to lead to mitotic delay which, over a wide range of doses, is proportional to the logarithm of the exposure. Similarly, sea urchin eggs exhibit a uv-induced mitotic delay which is exponentially dependent on dose.

Exposure of either the eggs or sperm of *Chaetopterus pergamentaceus* to γ-radiation results in a cleavage delay which is proportional to the logarithm of the dose. The sperm are significantly more sensitive than the eggs. This observation contrasts with our data on *Arbacia* which indicate identical sensitivities of the gametes at low doses and a hypersensitivity of the eggs at high ones. Ultraviolet irradiation of *Chaetopterus* sperm leads to a cleavage delay which is proportional to the logarithm of the dose. In contrast, the uv dose-dependence of the eggs is linear. There is a postfertilization increase in uv-sensitivity followed by a biphasic loss of sensitivity. Sea urchin eggs exhibit a similar pattern of sensitivity changes to γ-radiation; however, the uv pattern differs. There is a uv-sensitivity plateau followed by a linear loss of sensitivity. The differences between the responses of these two animals may provide a useful framework for the future analysis of the radiation sensitive mitotic events.

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Detection of changes of Ca_{in} *from* Limulus *ventral photoreceptors using arsenazo III.* G. WALOGA, J. E. BROWN AND L. H. PINTO.

Arsenazo III (K salt) was pressure-injected into *Limulus* ventral photoreceptors. Membrane voltage and the intensity of light (at 670 nm and 540 nm) passing through the single injected photoreceptor were measured simultaneously. With a bright, prolonged stimulus, the optical transmission at 670 nm (T_{670}) transiently decreased. The amplitude of the decrease in T_{670} depended on Ca_{out}. The transient decrease in T_{670} was detected in voltage-clamped cells and in cells containing 18 times more MOPS, pH 7.2 than dye, but was abolished when EGTA was iontophoretically injected into dye-injected cells. These findings indicate that light induces a transient rise of Ca_{in}, in agreement with findings of the aequorin technique. Since the increase in Ca_{in} lags light onset, the initial value of T_{670} indicates the resting level of Ca_{in}. To detect changes of Ca_{in} during changes of bath solutions, the intensity at 540 nm was subtracted from that at 670 nm; at these wavelengths, light-scattering changes have the same sign and were eliminated, but optical transmission changes due to the dye (T_{670}–T_{540}) have opposite sign and were detected. Using this technique, T_{670}–T_{540} progressively decreased during the iontophoretic injection of Na; the receptor potentials simultaneously were attenuated. Neither decrease was observed when K was injected iontophoretically. When the Na-pump was inhibited by bathing in zero K-SW or 10^{-6} M strophanthidin, both T_{670}–T_{540} and the amplitude of the receptor potentials gradually decreased. Also, when Li replaced Na in the bath, both T_{670}–T_{540} and the amplitude of the receptor potentials decreased; when the receptor potentials became smallest, immediately after the Li for Na replacement, the decrease in T_{670}–T_{540} was largest. These findings indicate that either increasing Na_{in} or decreasing Na_{out} leads to an increase of intracellular Ca, and are consistent with there being a Na-Ca exchange mechanism in ventral photoreceptors. The findings also are consistent with the hypothesis that a rise of Ca inside the photoreceptor leads to a decrease in the responsiveness to light.

Restrictions on translational diffusion of metarhodopsin in the membranes of a rhabdomeric photoreceptor. RÜDIGER WEHNER AND TIMOTHY H. GOLDSMITH.

In vertebrate photoreceptors, rhodopsin is free to undergo both rotational and translational diffusion in the disk membranes. For rhabdomeric photoreceptors, however, electrophysiological measurements of polarization sensitivity as well as microspectrophotometric evidence suggest that the rhodopsin molecules are not randomly disposed, implying restrictions on movement of the pigment. In order to ascertain whether translational diffusion is restricted, isolated, dark-adapted crayfish rhabdoms were examined from the side by microspectrophotometry. In layers of the rhabdom where microvilli extend at right angles to the direction of illumination, the closed ends of the microvilli of opposing cells abut along a central plane. As this plane represents membrane discontinuity, there should be no diffusion of pigment across it. Within such layers a local deficit of pigment was produced by a small bleaching spot. Photobleaching of crayfish metarhodopsin (λ_{max} 515 nm) was accomplished in 2 min at pH 9 in the presence of 0.25 M formaldehyde or glutaraldehyde. The decrease of pigment concentration within a reference spot (ca. 4×5 μm) located in the same layer of microvilli as the bleaching spot was independent of 1) whether reference or bleaching spots were lying on the same side or on opposite sides of the central plane, or 2) whether glutaraldehyde or formaldehyde was used for fixing the rhabdoms. Any loss of pigment in the reference spot was therefore due to light scattered from the bleaching spot and was not caused by lateral diffusion during the photobleaches and prior to remeasurement (up to 150–200 sec).

Following a local photobleach, the remaining metarhodopsin did not redistribute in the dark, but underwent a first-order dark decay, with the same rate constant at the bleaching and reference spots. The half-times in different rhabdoms varied from 15–25 min; within roughly this time there is therefore no evidence for translational diffusion of pigment.

Supported by the University of Zurich and USPHS grant EY00222.

Membrane perturbation: studies employing a calcium-sensitive dye (arsenazo III) in liposomes. GERALD WEISSMANN, TUCKER COLLINS, ALEX EVERS AND PHILIP DUNHAM.

A metallochromic dye, arsenazo III [2,7-bis-(2-arsonophenylazo)-1,8-dihydroxynaphthalene-3,6-disulfonic acid], which is potentially useful for the detection of changes in intracellular calcium activity, has been incorporated into liposomes. Capture of the dye in the aqueous interspaces of multilamellar liposomes (phosphatidylcholine 7: dicetyl phosphate 2: cholesterol 1) was established by the following criteria. Sepharose 4B chromatography was used to resolve liposome-associated dye (and glucose, a marker of the liposome aqueous compartment) from free dye and free glucose: 12 and 13% trapping of dye and glucose respectively were obtained with 32 μmoles of liposomal lipid/ml. Dye was latent within liposomes: detergent-ruptured liposomes yielded chromatographic evidence only of free dye and of glucose. Dye trapping was not electrostatic and varied with the molar percentage of dicetyl phosphate, the anionic component of the liposomes. Addition of 0.5 mM Ca^{++} produced no shift in the absorption spectrum of dye captured by liposomes (max, 560 nm, dye without Ca^{++}), whereas disruption of liposomes by Triton X-100 (0.5%, v/v), followed by Ca^{++}, produced the spectrum characteristic of the dye-C^{++} complex (maxima at 605 and 660 nm). Addition of 7.5 mM EGTA reversed the spectral shift. Differences between spectra obtained in this sequence yield *dye efflux*. To measure *Ca^{++} efflux*, difference spectra (±EGTA) were obtained from cationic liposomes containing Ca^{++} after detergent lysis (sensitivity < 10 nmoles/ml). By measuring extraliposomal dye-Ca^{++} complex, it was possible to test a variety of membrane-active steroids for their capacity to perturb lipsomes. Thus diethylstilbestrol, deoxycorticosterone, and etiocholanolone released 40.0, 26.3, and 25.8 nmoles of dye/μmole of lipid, respectively (*vs.* solvent controls of 10.9 nmoles of dye released), data comparable with more cumbersome and less sensitive methods. As in other systems, preincorporation of cortisol (molar lipid percentage: 1.0) stabilized liposomes to dye leak. Immunoglobulin-coated liposomes containing dye were taken up by phagocytes of *Mustelus canis*, and phagocytic vacuoles stained faint purple (dye without Ca^{++}) after ingestion. Liposomes containing the calcium-sensitive dye constitute a simple, accurate means for determining membrane perturbation and Ca^{++} fluxes, their uptake by cells or organelles remains to be exploited.

Differentiation of two histospecific enzymes in the same cleavage-arrested ascidian egg. J. R. WHITTAKER.

When fertilized eggs of the ascidian, *Ciona intestinalis*, were prevented from cleaving by exposure to 2 μg/ml cytochalasin B the zygote nucleus continued to divide throughout "development", resulting in large numbers of nuclei contained within a common cytoplasm. After appropriate times, such cleavage-arrested multinucleate eggs differentiated two histospecific enzymes: acetylcholinesterase (EC 3.1.1.7) of larval tail muscle and alkaline phosphatase (EC 3.1.3.1) ordinarily localized in the larval endoderm. Separate histochemical reactions on samples of eggs from the same animal provided 6 examples (from 34 animals) in which the percentages of the respective reactions overlapped sufficiently (15–29%) to suggest that some eggs have differentiated both enzymes in the same cytoplasm.

If there is a lineage of stable nuclear differentiation that occurs independently of local cytoplasmic influences and which a determinate cleavage pattern might be expected to orient and segregate approximately, then the cleavage-arrested eggs must contain many specialized nuclei in a common cytoplasm. Since regulatory products of these nuclei obviously do not repress one another's differentiated functions, the occurrence of these enzyme differentiations in cleavage-arrested eggs is contradictory to the idea of a nuclear lineage phenomenon in so-called mosaic embryos.

Development of each larval enzyme was prevented by puromycin treatment (200 μg/ml) applied just prior to the time at which each was first detected histochemically. Presumably the histochemical localizations represent new synthesis of the enzymes. It is not certain that both enzymes result from new genetic transcriptions; the alkaline phosphatase may be synthesized at least in part from maternal RNA. However, the above findings suggest that translational control mechanisms probably do not selectively (or competitively) determine the choice of differentiation pathways. Within the multinucleate single-celled embryo one phenotypic expression does not exclusively monopolize the translational system.

Supported by NIH grants HD–09201 and RR–05540.

On the mechanism of orientation towards water displacement sources in crayfish. K. WIESE.

Recently it was discovered that dually innervated, highly directional hairtype mechanoreceptors on the crustacean body surface match the cupula receptor in the lateral line organ in fish not only with respect to the dual innervation feature, but also in their sensitivity for water displacements and their effective frequency range. They are arranged in receptive fields which are within themselves homogenous in receptor-directionality, but differ in this respect from field to field. These findings induced me to perform the following experiment: a crayfish of 7 cm body length, anaesthetized by cooling for ½ hour on ice, was mounted in the center of a 30 cm large and 5 cm deep dish filled to half depth with icecold van Harreveld solution. The dorsofrontal carapax was opened and stomach and liver removed. A piece of black film underneath the oesophageal connectives improved visibility of single axons. After desheathing of the left side bundle single axons were rostrally disconnected and recorded from using suction electrodes. The water displacement stimulus was provided by a vibrating sphere (operating at 4, 6 or 8 Hz) which contacted the fluid surface. This wave source could be moved in 30° steps a full circle (17 cm diameter) around the preparation. A collection of 28 interneurons showed modulation of their response to a constant wave signal according to position of the wave source. The ranges of the individual maximal responses (30–60° wide) overlap to cover all angle ranges of the ipsilateral halfcircle. There are however also distinct heterolateral responses and fibers listening with equal intensity to both ipsi- and contralateral inputs. This observation is consistent with Wiersma's catalogue of connective fibers and their receptive fields. It is especially the hair-receptors on the walking legs which listen in the 90° and 270° direction. Inhibitory action from the contralateral homologous receptive field onto the recorded interneuron was observed in the claws and the right and left side of the telson.

I enjoyed the generosity of the Grass Foundation which provided me with a fellowship to perform this work.

The effect of forced cyclic gill movement on the respiratory rhythm of Limulus. GORDON A. WYSE AND CAROLYN H. MARKEY.

The gill plates of *Limulus* move in a metachronal respiratory rhythm of central nervous origin; the coordination and timing of this rhythm require no phasic sensory feedback. Gill plate movements, however, generate reafferent input and proprioceptive reflexes. This study examines the interaction of central and sensory timing information, and demonstrates that phasic sensory feedback from forced gill plate movements can extrain the centrally generated respiratory rhythm. The respiratory rhythm of gill ventilation was recorded with wire electrodes chronically implanted in gill plate muscles. The five pairs of gill plates were attached to a cam motor or to a load-compensated d-c linear actuator, so that they were all moved together at a direction and amplitude similar to the natural rhythm. Forced cyclic movement at a frequency near that of ongoing ventilation entrained the centrally generated rhythm, so that bursts of muscle potentials tended to occur at a preferred phase of the movement cycle. Phase histograms show that movement frequencies very close to the frequency of ongoing rhythm can completely entrain the rhythm (phase locking), while frequencies much faster or slower have no effect on the rhythm. For example, ongoing rhythm with an initial period of 2.0 sec was completely entrained by cyclic movement with 2.1 sec period, but drifted with respect to cyclic movement at 2.3 sec, with no preferred phase. Intermediate frequencies elicit phase preference or relative coordination. Sequential phase plots show that in such cases of phase preference, muscle bursts either (a) alternately phase lock and drift through the movement cycle, or (b) oscillate about the preferred phase. The temporal dynamics of this interaction between a central oscillator and reafferent timing information may facilitate analysis of mechanisms underlying the interaction.

Supported by NIH Grant NS-08869.

Subcellular localization of the antigrowth action of a purified sunlight-induced tryptophan photoproduct. S. ZIGMAN AND T. YULO.

Exposure of aqueous solutions of tryptophan (TRY) to sunlight in air or under several feet of sea water produces photoproducts that strongly inhibit mitosis and macromolecular synthesis in fertilized sea urchin eggs (*Arbacia punctulata*) at a level as low as 50 μg/ml. The near ultraviolet range (320–390 nm) of sunlight at a level of 2 to 4 mW/cm^2 at 365 nm produced such photoproducts within 3 hrs in 0.1% solutions of TRY in millipore-filtered sea water. Added to fertilized sea urchin eggs at 100 μg/ml, sunlight-exposed solutions of TRY in sea water doubled the time of the first cell division. The mitotic apparatus did not appear, and the first division produced abnormal daughter cells. When added later in development, subsequent mitoses were inhibited. Inhibition of the incorporation of ^{3}H-thymidine (TdR) into DNA and ^{14}C-amino acids (AA) into histones of chromatin by about 50%, and a 40% inhibition of ^{14}C-AA incorporation into ribosomal proteins, were observed at 45 min post-fertilization. Gel filtration and thin layer chromatography enabled the isolation of a TRY photoproduct ($PT2_{II}$) of high purity that enters sea urchin eggs and binds to chromatin, mitochondria, and ribosomes. Pure $PT2_{II}$ added to fertilized sea urchin eggs at 100–200 μg/ml strongly inhibits mitosis and synthesis of DNA and histones in chromatin. Inhibition of histone synthesis also resulted from the addition of $PT2_{II}$ to *in vitro* incubations of dogfish (*Mustelus canis*) cornea epithelial cells. A major action of the photoproduct $PT2_{II}$ is to interfere with histone-DNA relationships in chromatin required for normal cell division. Known chemical properties of $PT2_{II}$ are as follows: molecular weight, 300 daltons; UV absorption maxima, 315, 255, 225 nm; fluorescence excitation maximum, 310 nm, emission, 390 nm; Rf (butanol: HAc: H_2O, 4 : 1 : 1), 0.344; ninhydrin negative for free α-amino groups; and high water solubility. This photoproduct, produced in nature by sunlight or indoors by artificial UV light may be an important cell growth regulator.

This study was supported by the National Eye Institute and by ONR/CNA contract (Univ. of Rochester).

Continued from Cover Two

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CONTENTS

Bone, Q. and G. O. Mackie
Skin impulses and locomotion in *Oikopleura* (Tunicata: Larvacea). 267

Calder, Dale R.
Biotic census of Cape Cod Bay: hydroids.......................... 287

Fyhn, Unni E. H. and John D. Costlow
Tissue cultures of cirripeds.................................. 316

Hamby, Robert Jay
Heat effects on a marine snail................................ 331

Hassall, Mark and J. B. Jennings
Adaptive features of gut structure and digestive physiology in the terrestrial isopod *Philoscia muscorum* (Scopoli) 1763............ 348

Jones, Jack Colvard
Nonheterosexual behavior in mosquitoes.......................... 365

Lund, William A., Jr. and Barton C. Marcy, Jr.
Early development of the grubby, *Myoxocephalus aenaeus* (Mitchill) 373

O'Meara, George F. and John D. Edman
Autogenous egg production in the salt-marsh mosquito, *Aedes taeniorhynchus*.. 384

Stephens, Grover C.
Uptake of naturally occurring primary amines by marine annelids.. 397

Stross, R. G.
Causes of daily rhythms in photosynthetic rates of phytoplankton. II. Phosphate control of expression in tundra ponds............. 408

Abstracts of papers presented at the Marine Biological Laboratory...... 419

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THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. *A condensed title* or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

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THE

BIOLOGICAL BULLETIN

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INVESTIGATION ON THE ECOLOGY AND RESPIRATORY RESPONSES OF THE HEMICHORDATE *PTYCHODERA FLAVA* TO TIDAL CYCLES AND SALINITY CHANGES

Reference: *Biol. Bull.*, **149**: 455–466. (December, 1975)

JAYAPAUL AZARIAH, M. MOHAMED ISMAIL, AND M. AHMED NAJIB

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Different aspects of the biology of Hemichordates have been studied by a number of workers whose findings have been reviewed by Hyman (1959) and Barrington (1965). In recent years there have been some studies on the chemical constituents of Hemichordates (De Jorge, Petersen and Sawaya 1967; Ashworth and Cormier, 1967; De Jorge and Petersen, 1968a, b; Krishnan and Govindarajulu, 1968; Macha, 1969; Petersen and Longhi, 1971), but physiological aspects have received less emphasis. This may largely be due to "their sluggish responses and tendency to break into pieces on handling" which render them as "poor objects for laboratory experiments" designed to study their physiology (Hyman, 1959, p. 147). The paucity of physiological studies on Hemichordates prompted this investigation.

The occurrence of *Ptychodera flava* in the intertidal sheltered waters of Krusadi Island has been recorded by many workers (Ramanujam, 1935; Kuriyan, 1949; Sundara Rao and Ranga Rao, 1949). Rao (1954a, b; 1955a, b) studied some aspects of distribution, taxonomy, anatomy and development. Work on the physiology of Indian enteropneusts is hampered by their localized distribution around Krusadi Island and lack of good laboratory facilities there. It was, therefore, thought worthwhile to study some aspects of their respiratory physiology by transporting necessary laboratory equipment to Krusadi Island. Further, an attempt has been made to study their respiratory responses with special reference to salinity changes, since it is known (Jayaraman, 1954; Muthu, 1956; Udayavarma and Gangadhara Reddy, 1959) that there is a seasonal cycle in the salinity of the waters of the Bay of Bengal, with low salinity during the months of November and December. Some aspects of the ecology of *P. flava* are also considered in this paper.

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MATERIALS AND METHODS

Specimens of *Ptychodera flava* were collected from the lagoon area of the Galaxea reef during low tide by shoveling the sand gently with spread-out fingers. They were gently removed from the adhering mucous coat, cemented with sand, by repeated washing in sea water. Specimens were brought to the Krusadi Island Laboratory and kept under observation during the process of defecation. After 2.5 hours of collection, entire animals with their gut cleared of particles were used for experimental studies.

The apparatus used for the determination of oxygen consumption was a simple continuous flow system described by Fry and Hart (1948) and modified by Job (1955) and Azariah (1969; and unpublished). Sea water filtered with spun glass (Brosiam) wool and with maximum oxygen saturation was used. The animal (respiration) chambers were housed in an insulated water-bath, kept closed throughout the course of experiment in order to eliminate the influence of external factors. After 1.5 hours from the start of the experiment, half-hourly or hourly observations were made on solitary specimens. The volume of water flushing through the animal chamber per hour was calculated after every reading to arrive at the amount of oxygen uptake by the animal. Rate of flow did not influence the rate of oxygen uptake, but care was taken to keep the nozzle from clogging. Samples for oxygen content estimation were drawn from the outlet in bottles with approximate volumes of 15 ml. The initial oxygen concentration of water gaining entry to the respiration chambers was obtained from water-flushing a control respiration chamber without an animal. Oxygen analyses were made by the Winkler's method using N/100 sodium thiosulphate standardized against N/100 potassium dichromate. Experiments were conducted at $25 \pm 0.5°$ C using sea water of 30.5‰. Dry weights of the specimens were determined, and the rates of oxygen uptake were then calculated.

Individual observations were made on a total of 18 specimens at two different periods of the year. During January, 1974, experiments were performed on six animals; observations on three of these were related to the effect of salinity on the oxygen uptake. In the month of September, 1974, the animals were found to bear mature gonads, and hence only female specimens were chosen for the experiments as they were found in large numbers and easily distinguishable externally (Rao, 1954b).

In order to analyze the data statistically, the following procedure was adopted. The duration between a high tide peak and the succeeding low tide peak was divided equally. This was repeated for other high and low tide peaks. The sectors on either side of peak high tide and low tide were taken as the periods of high tide and of low tide, respectively. Data falling within such a sector were pooled, and the average was taken. The average values of all high tide and low tide periods during an experiment were added separately, and their mean represented the amount of oxygen uptake by an animal during the high or low tide period in the course of a single experiment. The values so obtained were used to illustrate the relationship between body size and oxygen consumption. Such a treatment may eliminate the influence of the phases of the habitat tidal cycle, if any, on oxygen uptake. The paired high tide and low tide averages of an animal were employed in the analysis of significance of variance using Student's t test.

In order to study the effect of salinity, the constant-level bottle was flushed with sea water of 20.5‰ salinity by opening the stopper in the tube connecting the reservoir containing the experimental medium (20 ‰) and closing the one in the reservoir with 30.5‰ salinity. The sea water in the constant level bottle was replaced within two minutes by increasing the inflow of water from the reservoir.

RESULTS

Ecological notes

Galaxea lagoon has water heights of about 2–3 feet during high tide, and less than 6 inches at low tide. *P. flava* leaves fecal castings on the surface of the sandy substratum, appearing as small hillocks of about 1.0 to 1.5 cm in height, with a centrally located burrow opening when submerged under water. The posterior anal end of the animal is seen protruding outside the substratum to a distance of about 0.5 to 3.0 cm more often than the anterior proboscis region. This makes the tail end opening of the burrow more prominent than the other. The proboscis is seen to extend outside the burrow up to the collar region. Freshly dug out animals are always covered by a thin mucous coat cemented with sand grains, which makes it difficult to distinguish them from the substratum. In the burrow, however, such a coat was not seen, and animals moved freely within their burrows. Secretion of a mucous coat may be an adaptation to avoid predation since it is reported that they are eaten by fish (Devanesan and Chacko, 1942) and found to lie on the substratum (Hyman, 1959).

Rao (1954b) reported the occurrence of *Edwardsia* (Anthozoa, Coelenterata) and sand-dwelling copepods, such as *Paramesochra arenicola, P. wilsoni* and *Emertonia minuta* (Crustacea, Arthropoda) in the area with *Ptychodera flava.* Forms like *Holothuria scabra* (Holothuroidea, Echinodermata) and hermit crabs in dead *Cerithium* shells have also been found. The area is dominated by *Cymadocea* sp. (sea-grass) with algae, such as *Acanthophera* sp., *Laurencia* sp., *Geladium* sp. and *Helimeda opuntioides* also being represented.

Ten acorn worms were taken to Madras (675 km distant) and were then reared in glass tanks containing 2–3 inches of sea water without sandy substratum. All were healthy and found to lie passively and extended, usually on their ventral side with the genital folds of either side meeting at the mid-dorsal line. The sea water was changed on alternate days. In the absence of sand the animals mainly depended on particulate matter for food and as a result a small amount of dark amber colored fecal matter was passed.

Nine acorn worms were then transferred to a glass tank containing an inch of sand collected from the inshore waters of Madras. On contact with the sand a slow wriggling movement of the body was observed in all the animals, with maximum activity in the proboscis region. As reported by Hyman (1959), there was copious secretion of mucus. Sand particles stream backwards from the proboscis region and get entangled in the thin film of mucus to form a tube-like structure around the animals. Formation of such a sand coat was continued until the region between the proboscis and a portion of the trunk region was covered. Then the proboscis was withdrawn inside the tube and did not make its appearance thereafter. Later, the withdrawal inside the tube is followed by deeper burrowing.

TABLE I

Relationship between body size, time taken for defecation and gut contents in P. flava.

Number	Breadth of collar (mm)	Time taken for defecation (minutes)	Weight of gut contents (mg)
1	3	30	240.4
2	5	45	293.6
3	5	25	360.0
4	5	95	358.0
5	5	90	389.6
6	10	95	1387.8

Table I shows the relationships between the size of the animal and the time taken for the gut to empty, together with the weight of the gut contents. It may be seen that the smallest specimen takes thirty minutes, and the largest takes about ninety-five minutes to empty its gorged gut.

Another facet of ecological interest is that of the differences between the pH of the sea water and the pH of the gut content. The pH of the sea water was between 7.0 and 7.2. After blotting, a fully fed animal was held vertically by the collar region and a portion of the trunk region was cut allowing free discharge of gut contents directly onto pH papers, which indicated a pH of 9.0. From the work of Barrington (1940) it is known that the amylase contained in body extracts was active between pH 5.5 and 8.0. The significance of the high alkaline pH in the gut compared to the near neutral pH of the habitat medium is not clear. Further work is proposed.

Rate of oxygen consumption as a function of body size and maturity stage

Figure 1 (solid squares) shows the relationship between the rate of oxygen consumption of six *P. flava* during the high tide period, (see materials and methods) and body dry weight; the animals range in body weight from 89.0 mg to 325.6 mg. The rate of oxygen uptake under constant conditions was found to vary between individuals and on the basis of weight, the smallest animal consumes 0.7629 ml/g dry wt/hr, which is about one and one-half times more than that of the largest. Individual variation in the amount of oxygen consumption of an animal, for example, 89.0 mg shows a wide range of variation from 0.0191 to 0.1056 ml/hr (Table III) which may be due to the correlation between the respiratory responses and phases of the habitat tidal cycle.

A linear regression equation calculated from the observed data (Y on x) gave the following regression coefficient: $Y = -521.99x + 469.46$, where $Y =$ the weight of the animal and $x =$ the rate of oxygen uptake.

Data obtained on the rate of oxygen consumption of twelve specimens of *P. flava* during the late maturity stage—mature and spawning—are presented in Figure 1. Data on low tide periods and high tide periods are plotted separately. In both the periods, the rate of oxygen consumption of the smallest animal is higher than that of the largest, and the increase is about six times during the low tide period and eight times during the high tide period. The range of individual variation in oxygen uptake during the course of each experiment is wide. The

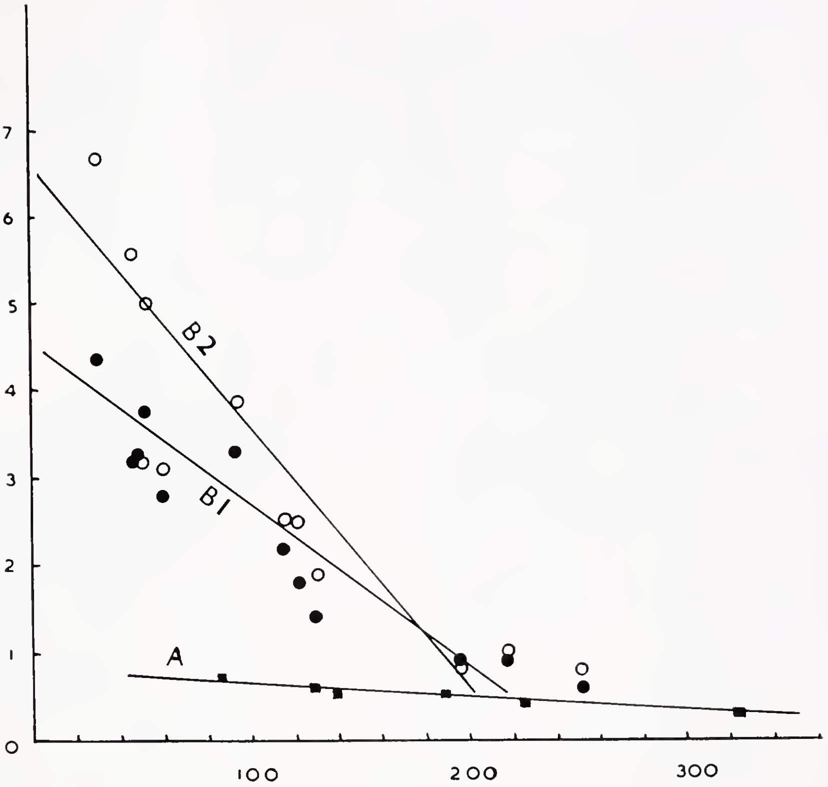

FIGURE 1. Rate of oxygen uptake of *P. flava* in relation to body weight; abscissa represents dry body weight in mg; ordinate, rate of oxygen uptake in ml/g/hr. Solid squares indicate the rate of oxygen uptake of *P. flava* during high tide periods in non-breeding season. Solid circles indicate the rate of oxygen uptake of *P. flava* during low tide periods in breeding season. Open circles indicate the rate of oxygen uptake of *P. flava* during high tide periods in breeding season. Solid lines A, B1, and B2 are the computed regression lines.

straight lines, B1 and B2, were derived statistically from low and high tide periods respectively; the regression equations being $Y = -53.63x + 244.78$ (B1) and $Y = -33.25x + 218.10$ (B2), respectively. The metabolic rate of the animals during the breeding season is higher than that of nonbreeding animals. The high tide values of the animals during both periods were subjected to Student's *t* test, and the difference between the two sets of values is statistically significant.

Oxygen uptake and tidal cycle

It is well known that periodic tidal inundations can shape behavioral and physiological responses into defined rhythms. Fluctuations in oxygen consumption over a period of time conform to tidal rhythm. Such results show three different patterns in the respiratory responses: T1, a neatly defined rhythm; T2, a tidal rhythm with additional peaks during low tides; or T3, absence of any defined rhythm correlated with phase of the tide. Data which are typical of the three

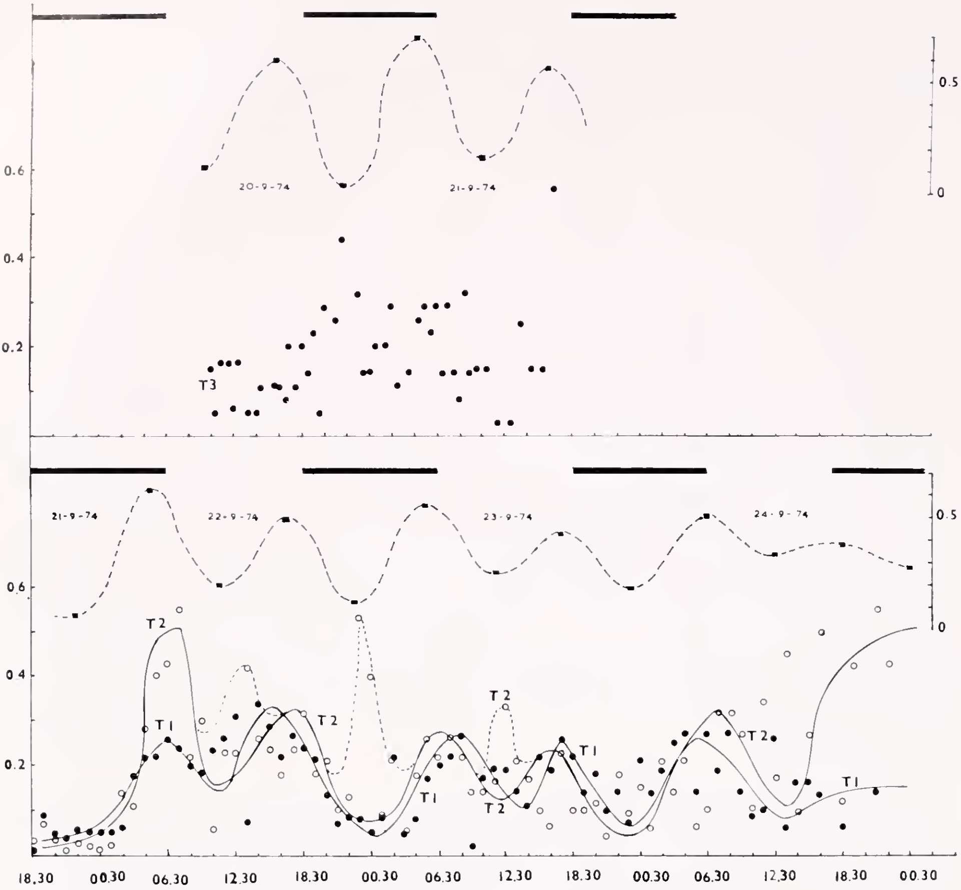

FIGURE 2. Three types of respiratory responses described in the text (T1, T2, T3) of *P. flava* to tidal cycle. The abscissae represent the times of day in hours; and the ordinates, rates of oxygen uptake in ml/hr. Solid bars represent hours of darkness in the habitat; and the ordinates in the upper right of each graph, the height of the tide in meters.

types of responses are illustrated in Figure 2, and for fifteen animals are given as the average values of oxygen uptake during the successive low and high tide periods in Table II.

Six out of fifteen specimens (T1) showed a rhythmicity in oxygen consumption whose phases seem to synchronize with some points of the habitat tidal cycle. The relationship between oxygen uptake and the tidal stages is direct, the rate of oxygen consumption being high during high tide and low during low tide.

In a further five out of the fifteen specimens (T2), besides the occurrence of a recurring rhythmicity, there was a peak value of oxygen uptake during low tide period. There remained four (T3) for which no rhythm could be detected.

Further, it is seen from Figure 2 that, when the height of the tide is lowest, the corresponding low tide values are also lowest when compared with other low

TABLE II

Data on the oxygen uptake of Ptychodera flava *during high tide and low tide: H represents high tide period; L, low tide period; Max, maximum amount of oxygen uptake, ml/hr; Min, minimum oxygen uptake, ml/hr; OAH, average oxygen uptake during high tide periods, ml/hr; OAL, average oxygen uptake during low tide periods, ml/hr; RAH, rate of oxygen uptake (ml/g/hr) during high tide periods; RAL, rate of oxygen uptake (ml/g/hr) during low tide periods. Dates of experiments for the animals were: 1 to 3 on January 23–24, 1974; 4 to 7, September 20–21, 1974; 8 to 11, September 21–24, 1974; 12 to 13, September 21–22, 1974; 14 to 15, September 22–24, 1974.*

	Oxygen uptake ml/hr														
Animal number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Dry weight (mg)	190.6	227.0	130.2	251.2	197.0	60.8	46.4	117.0	121.4	130.0	30.4	219.0	94.2	50.0	51.0
Tidal phase															
H	0.10012	0.10841	0.06927	—	—	—	—	—	—	—	—	—	—	—	—
L	0.07353	0.03545	0.07660	0.13765	0.28213	0.18570	0.07060	0.16613	0.09120	0.04478	0.04917	0.20132	0.19478	0.23610	0.11420
H	0.12466	0.07846	0.08880	0.13693	0.11264	0.16238	0.13001	0.37298	0.31395	0.32645	0.22468	0.25004	0.37145	0.20795	0.21506
L	0.03670	0.08183	0.18480	0.23580	0.15297	0.15844	0.21783	0.35076	0.34376	0.25812	0.21616	0.23653	0.33885	0.14976	0.22236
H	—	—	—	0.24875	0.20333	0.22037	0.44717	0.34050	0.34566	0.23988	0.26985	0.22060	0.36576	0.11895	0.29745
L	—	—	—	0.13597	0.13375	0.17602	0.16650	0.27171	0.29620	0.24137	0.10715	0.15650	0.44717	0.10747	0.25967
H	—	—	—	0.28337	0.17380	0.18630	0.21490	0.33000	0.26330	0.20080	0.17110	—	—	—	—
L	—	—	—	—	—	—	—	0.25543	0.14498	0.19993	0.13988	—	—	—	—
H	—	—	—	—	—	—	—	0.24520	0.20420	0.12650	0.20488	—	—	—	—
L	—	—	—	—	—	—	—	0.26518	0.21536	0.12708	0.14763	—	—	—	—
H	—	—	—	—	—	—	—	0.24900	0.32736	0.19706	0.23241	—	—	—	—
L	—	—	—	—	—	—	—	0.29376	0.22536	0.24473	0.15300	—	—	—	—
H	—	—	—	—	—	—	—	0.26076	0.39500	0.40730	0.12516	—	—	—	—
OAH	0.11239	0.09343	0.07903	0.22301	0.16325	0.18968	0.26402	0.29974	0.30824	0.24966	0.20468	0.23532	0.36860	0.16340	0.2562
RAH*	0.58910	0.41150	0.60690	0.88770	0.82860	3.11900	5.69000	2.56100	2.53900	1.92000	6.73200	1.07400	3.91200	3.26200	5.0230
OAL	0.05511	0.05864	0.13040	0.16980	0.18960	0.17338	0.14964	0.26716	0.21947	0.18600	0.13549	0.20010	0.32693	0.16444	0.19870
RAL*	0.28910	0.25830	1.00100	0.67590	0.96240	2.85100	3.22500	2.28300	1.80700	1.43000	4.45600	0.91360	3.47000	3.28800	3.89600
Min.	0.0205	0.01940	0.02050	0.03100	0.04880	0.07750	0.0132	0.04270	0.02440	0.00920	0.01320	0.05670	0.08710	0.03690	0.08970
Max.	0.2160	0.19220	0.13670	0.56680	0.44350	0.65010	0.4332	0.57450	0.51850	0.55470	0.34780	0.43340	0.70720	0.22950	0.49120

* Data used in Figure 1.

TABLE III

Influence of salinity on the oxygen uptake of P. flava; *the change from normal sea water (30.5‰) to subnormal sea water (20.5‰) took place between 1600 and 1630 hours on January 24, 1974. Data for the first three hours were also used in Figure 1. Mean (H) and Rate (H) refer to average oxygen uptake and rate of oxygen uptake during high tide periods.*

Time	Oxygen uptake ml/hr				Summary Oxygen uptake ml/hr		
	Animal 1 Dry wt 89.0 mg	Animal 2 Dry wt 140.0 mg	Animal 3 Dry wt 325.0 mg		Animal 1	Animal 2	Animal 3
13.00	—	0.0264	—		In normal sea water (30.5‰)		
13.30	0.1056	0.1608	0.1602				
14.00	0.0191	0.0191	0.0370	Mean (H)	0.0679	0.0811	0.1095
15.00	—	0.0864	—	Rate (H)	0.7629	0.5792	0.3363
15.30	0.0571	0.1075	0.0754	Min	0.0191	0.0191	0.0396
15.45	0.0756	0.0864	0.0935	Max	0.1056	0.1608	0.1814
16.00	0.0820	—	0.1814				
16.30	0.3067	0.1142	0.0972		In subnormal salinity (20.5‰)		
16.45	0.2102	0.2285	0.1085				
17.00	0.1401	0.1612	0.2277	Mean	0.1621	0.1358	0.1733
17.15	0.0969	0.0694	0.0874	Rate	0.1821	0.9700	0.5322
17.30	0.1180	0.2285	0.1404	Min	0.0643	0.0264	0.0217
17.45	0.1507	0.1080	0.1099	Max	0.3067	0.2285	0.3859
19.00	0.1392	0.0936	0.1837				
19.45	0.0643	0.0264	0.1814				
20.30	0.2958	0.1930	0.0630				
21.00	0.1366	0.0662	—				
22.00	0.1497	0.1242	0.0217				
22.45	0.1094	0.1613	0.2088				
00.00	0.1299	0.1005	0.3859				
1.00	0.2217	0.2261	0.2419				

tide values. The rhythm seems to be persistent for about 65 hours, and then becomes irregular or not detectable. Waning of the tidal rhythm was noticed in four animals studied for a period of 72 hours.

Salinity induced respiratory response

Data on the effect of salinity on oxygen uptake of three specimens are given in Table III. The average readings for three animals weighing 89 mg, 140 mg, and 325.6 mg in habitat sea water (30.5‰) are 0.0679, 0.0811 and 0.109 ml/hr, respectively and in reduced salinity (20.5‰) they consume 0.162, 0.136 and 0.173 ml/hr. The small specimen weighing 89.0 mg shows a difference of 0.094 ml/hr which is about 140% of the value obtained in habitat sea water; in the larger specimens, the increase being 66% (140 mg) and 58% (325.6 mg). It seems probable that the smaller animals respond more sharply than the larger animals. Response to salinity stress tends to become stabilized in a period of about an hour.

Discussion

The synchronization of one or the other of the physiological processes in intertidal animals with the phase of recurring tide can result in a rhythmical temporal pattern, the tidal rhythm. A tidal rhythm of oxygen consumption seems to persist in *Ptychodera flava* under constant conditions in the laboratory. Many factors could be involved in the actual entrainment, including: locomotor activity, diurnal periodicity, tidal inundation of sea water including temperature changes, mechanical agitation, and chemical changes in the medium. In *Ptychodera flava,* since the size of the animal chamber was just big enough to house the animal, which is sluggish most of the time (Hyman, 1959), it is concluded that the periodic increase and decrease in oxygen consumption could not be attributed to the locomotor rhythms and that the peak time of oxygen consumption must be due to other factors. A circadian rhythm may persist in the absence of daily cycles of temperature or of light, with maximum consumption of oxygen during the day, and minimum during the night. Specimens of *Uca* and *Sesarma reticulatum* show both circadian and tidal rhythms (Barnwell, 1966; Palmer, 1967) in that besides coincidence of two peaks with the high water, there is a prominent nocturnal peak of locomotor activity. In *Emerita,* an exaggerated nightly activity was considered to be due to the superimposition of a diurnal rhythm on a tidal cycle (Chandrasekaran, 1965). A close observation of the results reported in the present study reveals a decline in oxygen consumption at night. Concomitant increase in oxygen consumption with high water in the early hours of the day is also evident. All of this suggests a tidal rather than a true diurnal rhythm.

This rhythm reflects the prevailing ecological conditions in the habitat. In the area enclosed by the Galaxea reef, it is significant that the lack of breaking waves and the reduction of water depth results at times in the exposure of patches of the substratum. Any corresponding variation in the activity of the animals (by deeper burrowing, for example) will involve a variation in the pattern of oxygen uptake. The pattern of oxygen consumption in five out of fifteen animals is slightly at variance with the previous pattern in that there is an increase in oxygen uptake during the low tide periods.

Laboratory observations show that *P. flava* does make nocturnal excursions on the substratum, mostly when the conditions of the habitat become unfavorable in terms of the availability of food (Azariah, unpublished). They react to environmental extremes by migrating up to the surface and lying passively. Probably, in the environment, they may be moved by the waves to other more favorable places under these conditions. Thus, the activity patterns appear more complex and influenced by more than one factor.

Four animals did not show any tidal rhythm. The observations were made on the four animals simultaneously, and hence their pattern is a reflection of the pattern of the tidal cycle prevailing during the time of those particular experiments. The absence of tidal rhythm may result from the necessity for "conditioning" the animal to the experimental set-up used. In data obtained towards the end of the experiments (after about 20 hours), the animals show a tendency to exhibit a rhythm.

Enright (1963) reported that the activity levels of the amphipod *Synchelidum* were related to the amplitude of the tides. The activity rhythm of the sand crab *Emerita asiatica* coincided with the amplitude of the tide (Chandrasekaran, 1965). A similar relationship in *P. flava*, together with the waning of the tidal rhythms with time, suggests that the rhythm is phased by the environmental variables.

It is interesting to note the relationship between salinity changes and metabolism of *P. flava*. It responds to subnormal salinity by an increase in the respiratory rate, agreeing with the reports of Schlieper (1929), and Potts and Parry (1964). Although the increased respiration may suggest a causal relation to requirements for osmotic work, Potts and Parry (1964) and Vernberg and Vernberg (1972) suggest that changes in salinity may alter the locomotor activity of an organism, and hence changes in metabolic rate may reflect behavioral changes rather than the effect of salinity on basic metabolic processes (see Duncan, 1966). In *P. flava* no attempt was made to study its behavioral response to salinity changes. The pronounced rise in the respiratory rate on sudden changes of salinity and its gradual decline followed by the maintenance of a higher level of oxygen consumption than that of the one prior to exposure to a new salinity regime, show its short term overshoot reaction and the longer continuing responses. There seems to be a conflict between the inherent tidal rhythm and salinity-induced changes in respiration; there is a tendency to keep to the rhythm of tide, but the influence of salinity changes is dominant.

Time at Krusadi Island was limited, data were processed at Madras, and further experiments were impossible. Further work, following the suggestions given by Kinne (1971), is now planned.

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SUMMARY

1. Some aspects of the ecology and respiratory physiology of *Ptychodera flava* have been studied.

2. Increase in body weight involves a decrease in the rate of oxygen consumption. At high tides, the rates of oxygen uptake of nonbreeding *P. flava* are 0.7629 (smallest) and 0.3363 (largest) in ml/g/hr. At high tides, mature and spawning female rates are 6.732 (smallest) and 0.8877 (largest) ml/g/hr. Rate of oxygen uptake is a function both of body size and of maturity stage in *P. flava*.

3. Respiratory responses of 15 specimens of *P. flava* fell into three classes: 1, showing a rhythm synchronized with tidal phases; 2, showing a tidal rhythm with additional peaks during low tides; or 3, with no detectable rhythm.

4. *P. flava* reacts to lowered salinity by showing a sudden rise in respiration. The sustained level of respiration is subsequently a little higher than in normal sea water.

5. The deep-seated tidal rhythm shows variations, and it is suggested that locomotor activity, spawning, temperature, and salinity may all be factors involved.

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ULTRASTRUCTURE OF THE PIGMENTARY SYSTEM AND CHROMATOPHOROTROPIC ACTIVITY IN LAND ISOPODS

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Some of the most remarkable examples of physiological color change due to movements of pigment in chromatophores are found in crustaceans. In decapods, the production and release of substances with activity on chromatophores have been known since the first reports by Perkins (1928) and by Koller (1928). These chromatophorotropins are produced in the eyestalks, and Hanstrom (1933) first thought that the site of production was the sinus gland. It is now known that the sinus gland is merely a site for storage of these substances which are actually produced by another eyestalk structure, the X-organ. In fact, among others, Gabe (1967) could not obtain cytological evidence that the sinus gland has an incretory function. It does, however, maintain a close link with the X-organ through conducting axons of the X-organ's nerve cells which convey the secretion granules to the sinus gland (Passano, 1951, 1952; Bliss and Welsh, 1952; Bliss, Durand and Welsh, 1954).

The production of chromatophorotropins in isopods is an interesting problem to investigate, since physiological color change is reported lacking in terrestrial isopods and apparently also in freshwater forms (Buddenbrock, 1961, p. 291–292). The question for these species is whether the neurosecretory cells produce chromatophorotropins or whether lack of physiological color change is linked to ultrastructural changes in the pigment cells. McWhinnie and Sweeney (1955) reported that "larvae" of *Trachelipus rathkei*, a terrestrial isopod, show discrete chromatophores which later fuse in "syncytial nets"; adults of this species show weak color change in response to diffusely illuminated background, but chromatophore indices for such color change were not given. Several studies report chromatophorotropic potency of extracts of tissues from terrestrial isopods: Stahl (1938) found that head extracts of *Oniscus asellus* and *Porcellio laevis* contain a factor dispersing red pigment of Leander; Okay (1945) reported head extract of *Armadillidium* concentrates the dark pigment of *Idothea*, *Sphaeroma*, and *Ligia*. Finally, McWhinnie and Sweeney (1955), using isolated pieces of carapace of *Cambarus* as test objects, reported that crude extracts of *Trachelipus* sinus gland and nerve cord contained two antagonistic chromatophorotropins. However, the responses of *Trachelipus* itself to the extracts were not clearly established.

In marine isopods, the chromatophores are single stellate cells, in which the pigment granules can move to concentrate around the nucleus, or to disperse along the cytoplasmic processes. The mechanism of pigment granule migration in chromatophores remains obscure. The view that microtubules may in some way be involved in pigment migration, acting as a sort of active vector is derived from studies in anuran amphibians (Wise, 1969), teleost fishes (Bikle, Tilney and

Table I

Chromatophorotropic activities and pigment responses in land isopods.

Case	Donor	Structure	Receptor	Pigment	Effect	Author(s)
MI × MD	*Mesidothea*	head	*Leander*	red	disperse	Stahl (1938)
	Idothea	head	*Leander*	red	disperse	Carstam & Suneson (1949)
	Idothea	tail	*Leander*	red	disperse	Carstam & Suneson (1949)
TI × MD	*Oniscus*	head	*Leander*	red	disperse	Stahl (1938)
	Porcellio	head	*Leander*	red	disperse	Stahl (1938)
	Trachelipus	sinus gland	*Cambarus*	red	disperse	McWhinnie & Sweeney (1955)
	Trachelipus	nerve cord	*Cambarus*	red	concentrate	McWhinnie & Sweeney (1955)
MI × MI	*Ligia*	head	*Ligia*	dark	concentrate	Kleinholz (1937)
	Sphaeroma	head	*Sphaeroma*	orange	concentrate	Okay (1944)
	Sphaeroma	head	*Sphaeroma*	dark	concentrate	Okay (1945)
	Ligia	head	*Ligia*	dark	concentrate	Okay (1945)
	Idothea	head	*Idothea*	dark	concentrate	Okay (1945)
	Ligia	head	*Ligia*	dark	concentrate	Nagano (1949)
	Idothea	head	*Idothea*	dark	concentrate	Carstam & Suneson (1949)
	Ligia	sinus gland	*Ligia*	dark	disperse	Fingerman (1956)
	Idothea	head	*Idothea*	dark	disperse	Oguro (1959)
	Ligia	nerve cord	*Ligia*	dark	disperse	Fingerman (1956)
	Ligia	head	*Ligia*	dark	disperse and concentrate	Sawaya (1939)
	Ligia	head	*Ligia*	dark	disperse and concentrate	Enami (1941)
MD × MI	*Crangon*	eyestalk	*Idothea*	dark	disperse	Koller & Meyer (1930)
	Leander	eyestalk	*Idothea*	dark	disperse	Carstam & Suneson (1949)
	Cambarus	eyestalk	*Ligia*	dark	disperse	Enami (1941)
TI × MI	*Armadillidium*	head	*Idothea*	dark	concentrate	Okay (1945)
	Armadillidium	head	*Sphaeroma*	dark	concentrate	Okay (1945)
	Armadillidium	head	*Ligia*	dark	concentrate	Okay (1945)
TI × TI	*Trachelipus*	sinus gland	*Trachelipus*	dark	concentrate*	McWhinnie & Sweeney (1955)
	Trachelipus	nerve cord	*Trachelipus*	dark	disperse*	McWhinnie & Sweeney (1955)
MD × TI	*Cambarus*	eyestalk	*Trachelipus*	dark	disperse**	McWhinnie & Sweeney (1955)
MM × MI	*Praunus*	eyestalk	*Idothea*	dark	disperse	Koller & Meyer (1930)

MI = marine isopod.
MD = marine decapod.
TI = terrestrial isopod.
MM = marine *Mysidacea*.
* Possibly.
** Suggestive evidence.

Porter, 1966; Green, 1968; Wikswo and Novales, 1969; Castrucci, 1974a, b), and decapod crustaceans (Chassard-Bouchaud and Hubert, 1971; Elofsson and Kauri, 1971). Works in the field of isopod color change are summarized in Table I.

In view of the above facts, we investigated a series of both nonmarine and marine isopods to obtain more information about the alleged absence of physiological color change in land isopods and to find out whether this absence is related to (a) an ultrastructural alteration in their pigmentary system, (b) a lack of chromatophorotropic activity of the products of their neurosecretory cells, or (c) both.

Materials and Methods

Adult and young specimens of *Armadillidium vulgare, Porcellio laevis* and *Pardioniscus argentinus* were the land isopods chosen. The marine species *Ligia exotica* was used as test animal for injected homogenates of sinus gland and ventral nerve cord from the terrestrial forms, and in a morphological comparison of the pigmentary systems.

Electron microscopy technique

Pieces of tergites of the four species selected, in the intermolt stage, were fixed in 2% glutaraldehyde buffered with phosphate, pH 7.2, followed by 1% osmium tetroxide equally buffered. Tonicities were adjusted to 0.4 osmol for the land forms and to 0.99 for the marine species. The pieces were embedded in Cargille 6005 araldite. The 0.1 μ sections were double stained with 0.5% uranyl acetate and lead citrate. Pieces of the tergites, mounted in 70% glycerol, from animals fixed in 70% alcohol were used for optical observation.

Physiological technique

Homogenates of isolated sinus gland of the terrestrial isopods were prepared in filtered sea water in the following concentrations (glands/ml): 0.02; 0.20; 2.00; 8.00. The same procedure was used to check the presence of active principles in the ventral nerve cord homogenates, which had concentrations (cords/ml) corresponding to 2, 4 and 8. Sinus gland and nerve cord homogenates of *Ligia exotica* were also prepared for comparative purposes and blanks were made with sea water and distilled water. In order to break the secretion granules, the sinus gland and nerve cords, before homogenization in sea water, were ground in distilled water (1 ml). For homogenizing, 9 ml of sea water were finally added. The length of the receptor animals varied from 3.5 to 4.0 cm, and they received 0.03 ml of homogenate. The points in the graphs represent the mean degree of dispersion of melanophores of 3 sets of 10 receptors. Hogben and Slome's (1931) scale of dispersion was used. Homogenization followed the procedure used by Perez-Gonzalez (1957) for *Uca pugilator.*

RESULTS

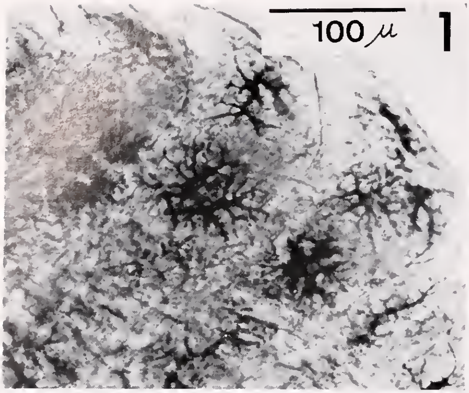
Responses of land isopods to environmental changes

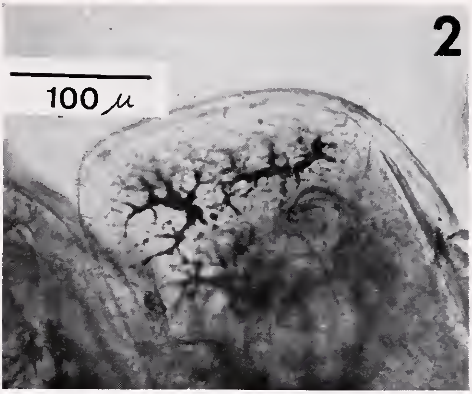
In order to test the responses of the three land isopod species to environmental changes, they were submitted to the following conditions: (a) total darkness, (b) illuminated black background, and (c) illuminated white background. In 2-hour experiments, the animals showed no changes in color. In parallel experiments with *Ligia exotica,* there occurred the typical responses of this marine isopod; that is, darkening in complete darkness or on an illuminated black background and blanching on an illuminated white background. Just-pigmented young of the three land species submitted to similar conditions also showed no color change.

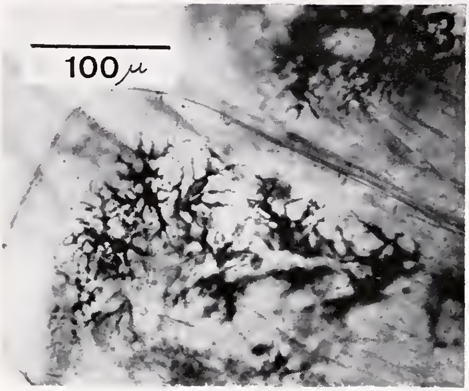
Light microscopy of the pigmentary system of land isopods

Adults of the three species studied showed no individual chromatophores, but did show an apparently syncytial pigmentary system, containing dark brown granules.

Only the young of *P. argentinus* on release from the marsupium show body pigmentation; the young of both *A. vulgare* and *P. laevis* exhibit pigmentation







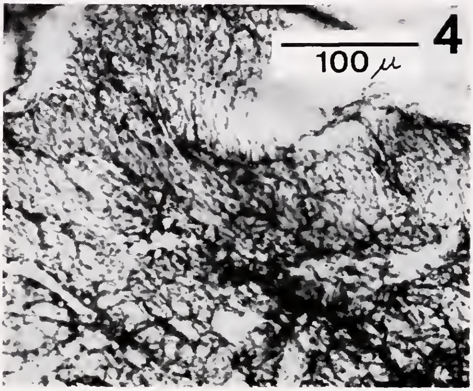


FIGURE 1. Chromatophores of *Pardioniscus argentinus* just released from the pouch.
FIGURE 2. Beginning of pigmentation in *Armadillidium vulgare* (age 21 days).
FIGURE 3. Beginning of pigmentation in *Porcellio laevis* (age 26 days).
FIGURE 4. Pigmentary system of *Armadillidium vulgare* (adult).

only in the ommatidea. As shown in Figure 1, single chromatophores exist on the edges of the carapace of *P. argentinus* just liberated from the pouch, whereas body pigmentation after liberation starts approximately on the 21st day in *A. vulgare* (Fig. 2) and on the 26th day in *P. laevis* (Fig. 3). In the three cases, the single chromatophores grow in number and apparently fuse in a syncytial network with time, as shown in Figure 4 for *A. vulgare*.

Electron microscopy of the pigmentary systems

In all of the terrestrial forms studied, there is a layer of typical epithelial cells under the chitin, overlying the very thin pigmentary network. This network is in fact formed by contiguous stellate elements whose arms spread under the epithelial layer and contain electron-dense pigment granules. These granules are round and possess a single membrane. The elements have a unit membrane; and, where the arms of two elements contact, there is a very thin layer of connective tissue in the

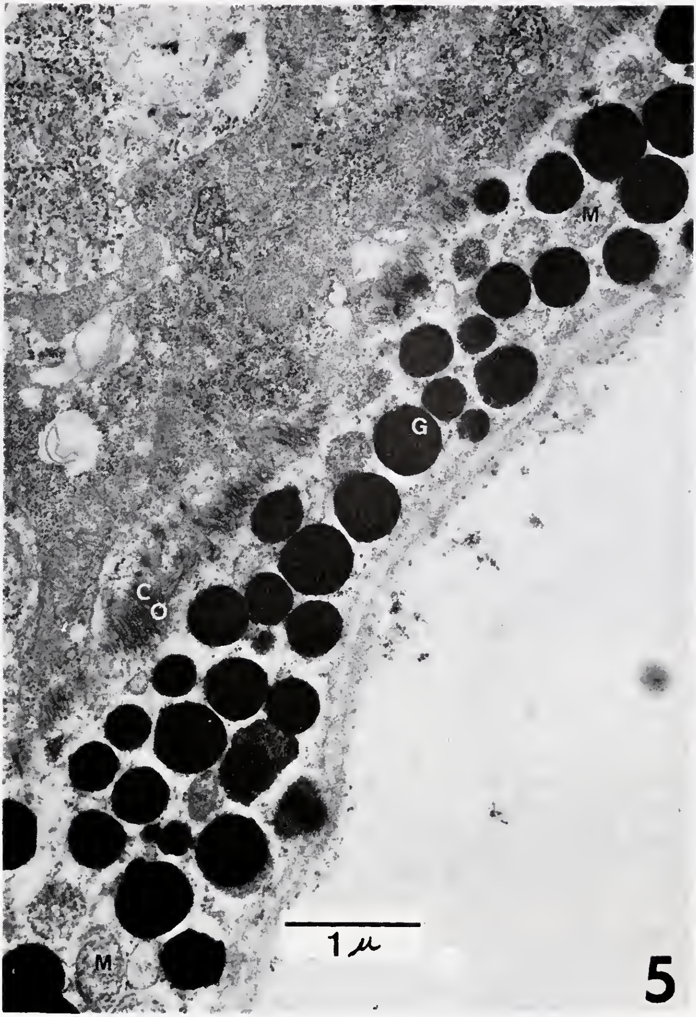


FIGURE 5. Branch of an element of the pigmentary system in a land isopod (*Porcellio laevis*): G indicates pigment granules; M indicates mitochondria and CO indicates collagen.

space between. The cytoplasm of the elements contains mitochondria, and little smooth endoplasmic reticulum, but no ribosomes or microtubles (Fig. 5).

In contrast with land isopods, the marine forms possess individualized chromatophores (melanophores), in which there are a rich smooth reticulum, microfilaments and microtubules. Along the cell processes (Fig. 6), the microtubules occur largely among the pigment granules and the reticulum membranes.

Neurohaemal structure in land isopods

In the distal third of each optical lobe in the three species studied, there is a stalked vesicle attached to the nervous structure. The topography and the histo-

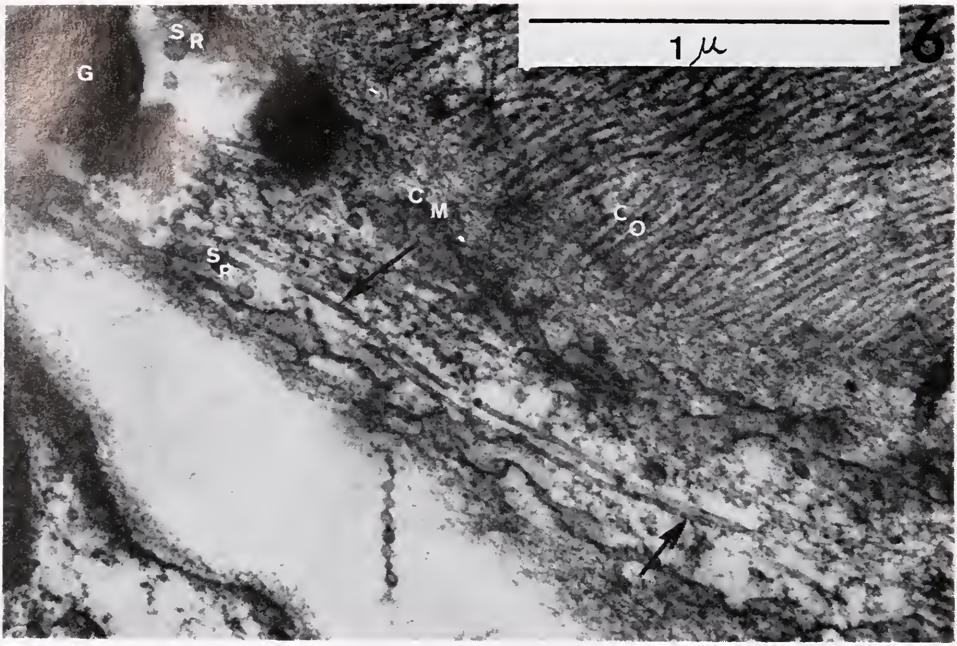


FIGURE 6. Cellular process of a chromatophore in a marine isopod (*Ligia exotica*): G indicates pigment granules; SR shows smooth endoplasmic reticulum; CM indicates cell membrane and CO indicates collagen. The arrows show microtubules.

logical features of this vesicle allow it to be considered equivalent to the sinus gland of decapod crustaceans; that is, a neurohaemal organ acting as the site of hormone storage. Figure 7 shows the structure of this vesicle, which we used to make the homogenates.

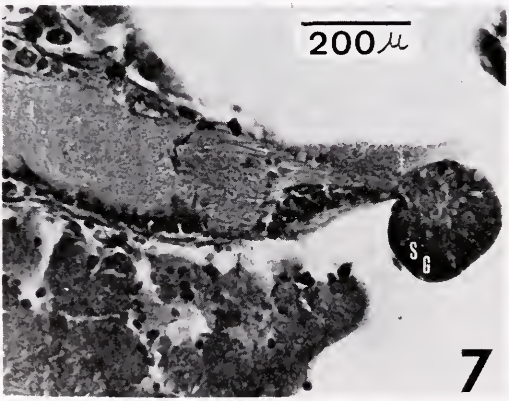


FIGURE 7. Sinus gland of a land isopod (*Armadillidium vulgare*): SG indicates sinus gland.

Chromatophorotropic potencies of whole head or nervous structure homogenates of land isopods

At first, whole head homogenates of *A. vulgare*, *P. laevis* and *P. argentinus* were injected in *Ligia exotica* adapted to a white or black background. They had a strong dispersing effect in the melanophores of the marine form; no concentration was observed. The injection of head homogenates of *L. exotica* in *L. exotica* adapted to a white or black background caused only dispersion. However, the suspicion that the observed results could be due to other causes (for instance, the osmotic effect of nonactive tissues of the head) lead us to a more careful determination of specific neurosecretory activity, using isolated sinus gland and nerve cords.

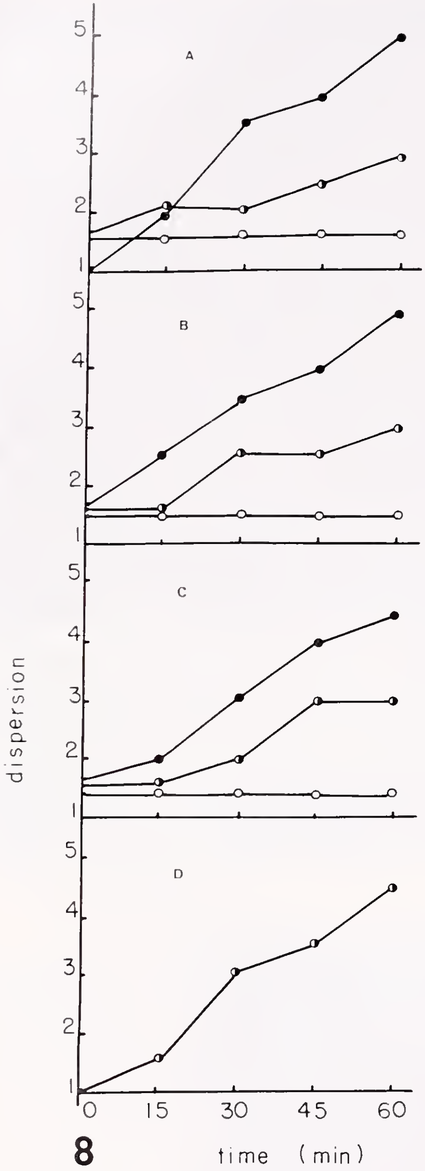
The graphs of Figures 8 and 9 refer to the action of homogenates prepared respectively with excised sinus glands and nerve cords of the land isopods on melanophores of *L. exotica* adapted to a white background. On a gland/ml basis, 0.02 or 0.2 homogenates of *A. vulgare* had no effect; 2.0 homogenates caused, within one hour, a pigment dispersion up to stage 3; and 8.0 homogenates induced maximal dispersion within the same time. Similar results were obtained with sinus gland homogenates of *P. laevis* and *P. argentinus* (Fig. 8). In all cases the homogenates caused no concentration of the pigment in the melanophores of *L. exotica* adapted to a black background. In control experiments, homogenates of the optical lobe of *L. exotica* caused strong dispersion, but not concentration of the melanophore pigment of the same marine isopod.

The graphs of Figure 9 show the action of homogenates, made with nerve cords from the land isopods, on *L. exotica* melanophores. On a nerve cord/ml basis, 2.0 homogenates were ineffective; but 4.0 and 8.0 homogenates elicited pigment dispersion in proportion to concentration. However, in the case of homogenate from *L. exotica* injected in *L. exotica*, 2.0 homogenates were enough to induce dispersion of pigment granules. In no case did nerve cord homogenates promote a pigment concentration in *L. exotica* melanophores.

Discussion

The *leitmotif* of the present study was to investigate the lack of physiological color change in land (and freshwater) isopods and to find out (a) to what ultrastructural change is it linked, and (b) in what measure is it related to a loss (or decrease) of chromatophorotropic potency of the neurosecretions of these animals.

McWhinnie and Sweeney (1955) studied the chromatic behavior in the land isopod *Trachelipus rathkei*, following the suggestion of Walker (1935) and Stahl (1938) that other nonmarine isopods should be investigated in search of chromatophorotropins, since a structure homologous to the decapod's sinus gland was found in *Oniscus asellus*. McWhinnie and Sweeny's paper is perhaps the sole reported investigation dealing specifically with physiological color change in nonmarine isopods. Yet, it is not quite clear whether or not *T. rathkei* really changes color in response to background. Adults exhibited weak and slow responses to light; in young ("larvae"), the pigment in the discrete chromatophores concentrated under light stimulus. McWhinnie and Sweeney (1955, p. 173) did not particularly approach the question of the lack of physiological color change



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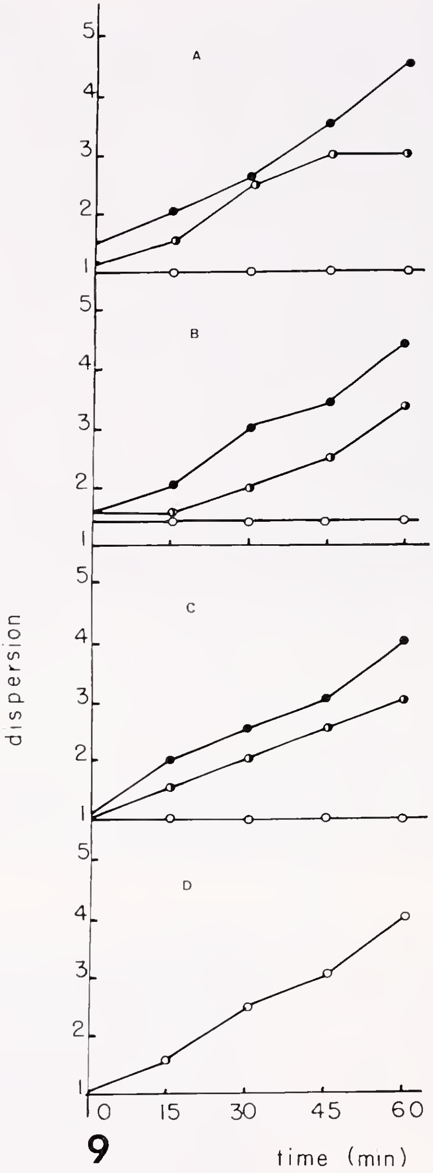

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FIGURE 8. The effects of injections of sinus gland homogenates of *Armadillidium vulgare* (A), *Porcellio laevis* (B), *Pardioniscus argentinus* (C), and of optical lobe homogenate of *Ligia exotica* (D) on melanophores of lightened *Ligia exotica*. Open circles indicate effect of 0.02 and 0.2 glands/ml homogenates; half-filled circles indicate effect of 2.0 glands/ml homogenates; and filled circles indicate effect of 8.0 glands/ml homogenates.

FIGURE 9. The effects of injections of nerve cord homogenates of the same four species (A-D) on melanophores of lightened *Ligia exotica*. Open circles indicate the effect of 2 cords/ml homogenates; half-filled circles indicate the effect of 4 cords/ml homogenates; and filled circles indicate the effect of 8 cords/ml homogenates.

in land isopods, but they admitted that "the loss of reactivity of the pigmentary system is a function of the progressive fusion of the chromatophore network, with age." In our experiments with the three species studied (and additionally, with two other land species, *Porcellio dilatatus* and *Benthana picta* and a freshwater form, probably of the genus *Ianirops*) no color change could be induced by presence or absence of light, black or white substrates, in either adults or young.

McWhinnie and Sweeney (1955) admitted that in *T. rathkei*, the chromatophore processes fuse with age in a true syncytium. However, the electron microscopy revealed that, in the species we investigated, this syncytium is only apparent and the pigmented processes are merely contiguous. The electron micrographs revealed another important thing: in comparison with the processes of the true melanophores of the marine isopod, no microtubules or microfilaments could be visualized in the branches of the land isopods network, and reticulum membranes are less frequent. This structural difference may be of great importance in connection with the lack of pigment migration in nonmarine isopods, since it has been suggested that, in some way, these structures may be essential organelles for pigment movements in true chromatophores.

On the other hand, the tests of chromatophorotropic potencies in land isopods, using isolated neurohaemal and neurosecretory structures, indicated that the morphological change in the pigmentary system was not accompanied by the loss of capacity to produce chromatophorotropins. The acceptance of the stalked vesicle found along the optical lobe as corresponding to the sinus gland is based on similar findings by McWhinnie and Sweeney (1955) in *Trachelipus rathkei*, Matsumoto (1959) in *Armadillidium vulgare*, and Vitez (1970) in *Porcellio dilatatus*, *Porcellio laevis* and *Protracheoniscus asiaticus*. Actually, the question of the homology between neurohaemal and neurosecretory sites in isopods, and the sinus gland and the X-organ in decapods, remains open to discussion. For instance, in de Hureaux's paper (1967) it is not clear which is the Bellonci's organ (equivalent to the sensory pore X-organ of decapods) and which is the sinus gland; in the text, the Bellonci's organ would be the organ considered here as the sinus gland; whereas in the figure of the dissected nervous system, the structure designated as the sinus gland corresponds also to our sinus gland. Our homogenates showed chromatophorotropic activity as did the extracts prepared by Pigeault (1958) and by de Hureaux (1967) from *Sphaeroma serratum* Bellonci's organ. A ganglionar X-organ does not exist in isopods, according to Messner (1966). However, as the axons of cells can be followed to the sinus gland (Vitez, 1970), it is possible that their secretion is stored in the neurohaemal organ. Thus, the cells could be considered as a simplified ganglionar X-organ.

Other questions refer to whether in isopods there occur two chromatophorotropins and to what is the role of each. The presence of a second and antagonistic hormone controlling the melanophores of isopods was suggested by Smith (1938), working with *Ligia exotica*. In this animal, background responses were shown to be dependent upon two antagonistic hormones, one inducing dispersion and the other causing concentration of melanin. Table I shows some of the conflicting results of experiments in which decapods, isopods and even a *Mysis* genus were combined as donor and receptor of chromatophorotropins. An inspection of this table reveals that: (1) the dispersion of the red pigment of marine macruran decapods was consistently obtained with material from both marine and terrestrial isopods; (2) in some cases head extracts from marine isopods concentrated, in others they dispersed the dark pigment of marine isopods, and in two cases these extracts contained both dispersing and concentrating principles; (3) extracts from marine macruran decapods (group I, "*Palaemonetes* group") dispersed the dark pigment of a marine isopod; (4) in three marine isopods, head extracts from a land species concentrated the dark pigment; and (5) in a single instance, material from a land isopod and from a macruran decapod was used in a terrestrial form, but due to the uncertainty as to a real color change in the receptor, the results were considered as merely suggesting the presence in the land isopod of a concentrating substance in the sinus gland and a dispersing one in the nerve cord. Our results favored acceptance of only the dispersing factors in both sinus gland and ventral nerve cord as in Fingerman's reports for *Ligia* (1956) and Oguro's for *Idothea* (1959).

Thus, the results reported in this paper support the idea that in land isopods, in spite of the lack of physiological color change, chromatophorotropic substances are still produced by neurosecretory cells of the cerebral ganglia and of the nerve cord; at least one of these dispersing chromatophorotropin. The genetical change which induced the conversion of individual chromatophores of the marine species into the network of the land isopods (in which the melanosomes are motionless and no microtubules or microfilaments occur) was not paralleled by the supression of chromatophorotropin production. The real role of microtubules on pigment migration is yet to be determined. Various workers who have treated chromatophores with microtubule disrupting drugs have not yet obtained conclusive results. In fishes, Wikswo and Novales (1969) reported centripetal movement-inhibition in *Fundulus* melanophores by colchicine treatment, while Castrucci (1974b) obtained (in *Tilapia* chromatophores) inhibition of both centripetal and centrifugal movements by colchicine or vinablastine treatment. In anurans, Malawista (1971) found increased pigment dispersion and decreased pigment aggregation in frog melanocytes treated with colchicine. Though these reports could suggest that microtubules are required for pigment movements, the findings of Robison and Charlton (1973) pointed out the important role of microfilaments on pigment migration. These authors did not succeed in obtaining any alteration of pigment movements in chromatophores of *Palaemontes* after incubation with colchicine or vinblastine. However, using cytochalasin B, which disrupts the microfilament pattern, pigment aggregation was reversibly inhibited. Thus, in this species, pigment aggregation would be more related to filament array than to microtubule integrity. In the case of isopods, our results indicate that the terrestrial forms differ from the marine ones not only in the configuration of the pigmentary system and lack of color change but also in that no microtubules or microfilaments were seen in the pigment cells. This fact supports the possibility that both organelles are needed for pigment displacement.

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SUMMARY

1. The question of the absence of physiological color change in terrestrial isopods was investigated with three species of land isopods (*Armadillidium vulgare, Porcellio laevis* and *Pardioniscus argentinus*) and a marine species (*Ligia exotica*) as reference.

2. The young and adults of the terrestrial isopods do not change color when exposed to dark or light conditions, nor to white or black backgrounds.

3. Light microscopy showed that the pigmentary system of the terrestrial isopods is an apparently syncytial net work containing a dark brown pigment.

4. Electron microscopy revealed that the pigmentary network is only apparently syncytial; its elements are merely contiguous. As compared to chromatophores of *Ligia*, the elements of the network of land isopods are much poorer in smooth endoplasmic reticulum and have no microtubules.

5. Homogenates of sinus gland and of nerve cord from land isopods induced pigment dispersion in melanophores from *Ligia exotica;* the effect was proportional to concentration of homogenate. Such extracts had no effect on melanophores of darkened animals.

6. The results obtained with the terrestrial species indicate that these isopods can produce chromatophorotropic-active substances (pigment-dispersing principles) despite their lack of physiological color change.

7. The lack of physiological color change is associated with the disappearance or absence of microtubules and reduction of endoplasmic reticulum, although there is no substantial evidence that these organelles play a role in pigment migration.

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THE MATERNAL POUCH AND DEVELOPMENT IN THE MARSUPIAL FROG *GASTROTHECA RIOBAMBAE* (FOWLER)

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Gastrotheca riobambae (Fowler) is a common frog in the northern interandean valleys of Ecuador. In this genus, after the eggs leave the female's cloaca, the male places them inside a dorsal pouch in the body of the female (Walker, 1957; Matthews, 1957) where the embryos develop until the tadpole stage. Spannhof and Spannhof (1972), working with *Gastrotheca marsupiata,* found that incubation of embryos in the pouch lasts approximately five to six weeks, but the larvae may remain in the pouch for considerably longer periods, depending on environmental factors. After leaving the pouch, the tadpoles continue their development in water, where metamorphosis occurs in a few weeks. Hoogmoed (1967) has studied the mating and early development of *Gastrotheca marsupiata* and reviewed most of the existing literature. Additional observations on the embryonic incubation and larval development of *Gastrotheca marsupiata* have been reported by Spannhof and Spannhof (1972). Recent studies of the taxonomy and distribution of *Gastrotheca* in South America resulted in the conclusion that the populations of this genus in the vicinity of Quito are primarily *Gastrotheca riobambae* (Duellman and Fritts, 1972; Duellman, 1974). Although reports dealing with the pouch and reproductive activity of *Gastrotheca riobambae* are limited to the paper of Jones, Gerrard and Roth (1973), it is possible that some descriptions, such as those of Hoogmoed (1967), are actually based on *G. riobambae,* rather than *G. marsupiata,* since the animals were collected near Quito.

The maternal pouch of *Gastrotheca* is of uncommon interest as an adaptation to life on land, but surprisingly little is known about it. Experimental induction of pouch formation in juvenile females of *Gastrotheca riobambae* has been studied (Jones *et al.,* 1973), and aspects of the histology of the pouch of *Gastrotheca marsupiata* have been described (Spannhof and Spannhof, 1972); but the details of the histology and physiology of the pouch and the processes of embryonic development in the pouch have not yet been thoroughly studied. In this report we present the initial results of a study of reproduction in *Gastrotheca riobambae*, with particular emphasis on the structure of the pouch and embryos, maternal-embryonic relationships and changes in the pouch related to reproductive activity.

MATERIALS AND METHODS

Adult specimens of *Gastrotheca riobambae* were collected in Quito, Ecuador, on the grounds of Pontificia Universidad Catolica del Ecuador, from other localities within the city, and near Machachi, a town located approximately 50 km south of Quito. The animals were brought into the laboratory and kept in a 1.50 × 0.40 ×

0.60 m terrarium. The terrarium was provided with vegetation and a tray containing pond water and several stones which gave supporting surfaces for the females at the time of tadpole hatching and emergence from the pouch. Both brown and green variants of the species were common at the collection sites and frogs of both colors were used for this study. Observations are based on a total of more than 50 frogs.

Ovulation was induced by the administration of 800 IU of human chorionic gonadotropin (Coriantin, Richter) into the coelomic cavity of the female. Males were stimulated to mate by the similar administration of 100 to 200 units. Mating occurred 24 hours or more after hormone administration. As stated, mating occurs on land, and, as they emerge from the cloaca, the eggs are moved into the pouch by the male. Sometimes only the female responded to the hormone treatment and in such cases the eggs released were not placed in the pouch but instead were deposited and left on the ground.

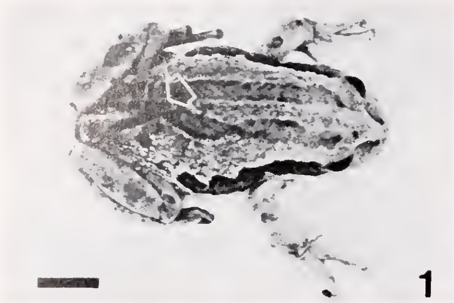
Pouch and embryos were fixed in Bouin's picro-formol, embedded in paraffin and cut into sections of 10 μm thickness. Harris hematoxylin and alcoholic eosin yellow were used for routine staining. Whole mount permanent preparations of embryos were made by cutting the embryos from the yolk, fixing them in Bouin's and staining them with borax-carmine.

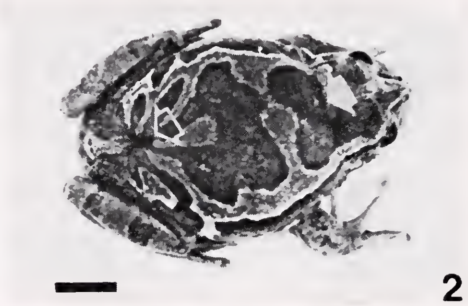
Eggs and embryos from the pouch were cultured in pond water or in several concentrations of amphibian Ringer's solution.

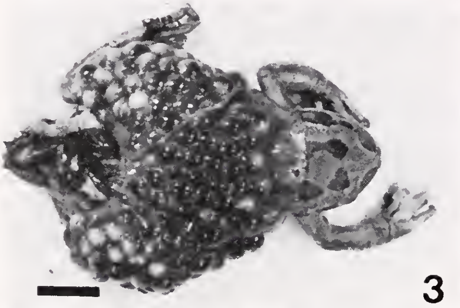
RESULTS

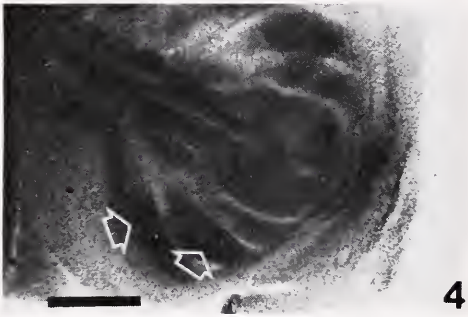
General morphology of the pouch

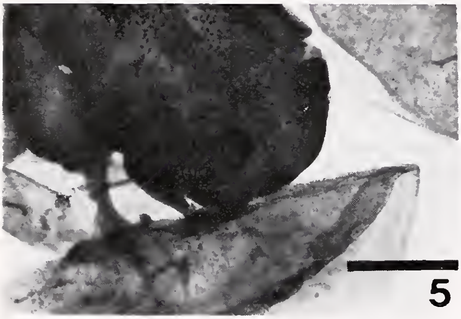
The pouch is a sac underlying the dorsal integument, but is essentially independent of it except at the aperture. Histologically, the pouch resembles amphibian integument, but differs significantly from it, particularly during reproduction. The pouch is absent in males and juvenile females, but is always present in the sexually mature female. The aperture of the pouch is triangular, with the apex of the triangle directed anteriorly (Fig. 1). The entrance to the pouch can be open or closed, depending on the stage of the reproductive cycle. In open pouches, the borders of the aperture are wide apart, giving a broad triangular or "U" shape (Fig. 1), whereas in the closed condition the borders of the aperture join in the midline of the body, giving a slit-like appearance (Fig. 2). The pouch aperture remains open after tadpole birth and throughout the growth of the next generation of oocytes in the ovary. Closure ordinarily occurs when the ovarian eggs have attained their full size just before the time of ovulation, but in females with large ovaries, closure can be induced experimentally in about twelve hours by injection of chorionic gonadotropin. The pouch remains closed during the time the embryos are being incubated, but even during this period it is easy to gain access to the pouch with a blunt probe or forceps for the removal of embryos. Under ordinary conditons, opening of the pouch occurs when the tadpoles are ready for hatching and release; we have observed, however, that handling of a frog with a closed pouch results in an immediate opening of the pouch. Following such an opening, if the animal is left undisturbed, the aperture closes again in about an hour.











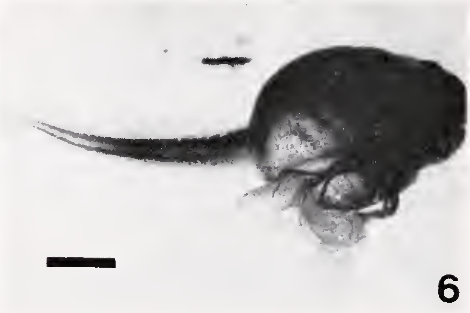


FIGURE 1. Female *Gastrotheca riobambae* before mating. The pouch aperture is open. Pouch aperture is triangular in shape, with the apex of the triangle (arrow) directed anteriorly. Bar represents 1 cm.

FIGURE 2. Female with developing embryos in the pouch. The pouch is considerably distended, with its anterior limit reaching almost to the head (solid arrow). The aperture of the pouch is closed and slit-like (open arrow). Bar represents 1 cm.

FIGURE 3. Female with developing embryos in the pouch. The dorsal integument has been removed to expose the intact distended pouch with the embryos inside. The pouch is almost transparent and fills the entire dorsal and lateral sides of the body. Bar represents 1 cm.

FIGURE 4. Early embryo removed from the pouch. The yolk was dissected and the embryo was prepared as a whole mount. The primordia of the bell gills are noticeable on both sides of the head (arrows). Bar represents 500 μ.

FIGURE 5. Advanced embryo removed from the pouch and jelly capsule (whole mount permanent preparation). The bell gills are large discs that in the living condition completely enveloped the embryo. Each gill is connected to the body by means of gill stalks (arrow). Bar represents 500 μ.

The pouch is attached to the integument and to the part of the body lying ventral to the pouch by thin lateral ligaments carrying blood vessels and nerves. In addition, there are long thread-like structures lying in the midline, carrying vessels and possibly nerves, which connect both the ventral and dorsal walls of the pouch with the area ventral to it. There is a sheet of muscle on the dorsal side, extending between the integument and the pouch. Although the pouch is a permanent structure, it varies considerably in size, depending upon the phase of reproduction. In nonpregnant females (*i.e.*, females without eggs in the pouch), the pouch extends approximately 1 cm anteriorly and laterally from the aperture. The pouch of the pregnant female becomes highly distended as the embryos develop, extending approximately 3 cm anteriorly from the aperture and bringing the limits of the fully-distended pouch anteriorly to the base of the head and laterally into the ventral portions of the body (Figs. 2 and 3). The distension of the pouch at this time is such that its walls become almost completely transparent (Fig. 3), in contrast to the essentially opaque condition of the nonpregnant pouch.

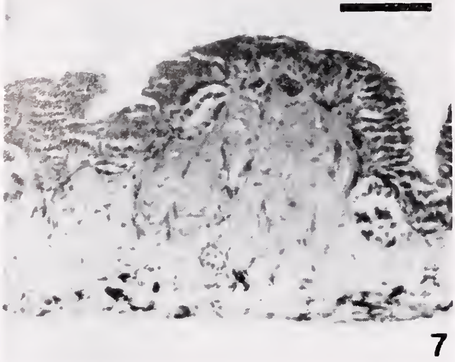
Structure of the nonpregnant pouch and changes during reproduction

The histological structure of the pouch before ovulation resembles that of amphibian integument. The pouch is lined with stratified squamous epithelium, which is closely associated with numerous simple alveolar mucous glands, usually with large lumina. Some glands have small lumina, and the cells of these glands are considerably swollen with accumulated secretory material. A few scattered serous glands are present. As compared with the skin, the pouch contains fewer mucous and serous glands, and its epithelium is thrown into numerous folds at this stage (Fig. 7). The pouch epithelium also appears less keratinized than the epithelium of the skin. Just below the basal membrane, chromatophores and sometimes small blood vessels are seen. The corium is thick, with scattered large blood vessels located deep within it. The part of the corium immediately below the basal membrane is loosely arranged and serves as a matrix for the glands, while the deeper layer contains dense fibers of connective tissue in parallel bundles, as well as some elastic fibers. A layer of muscle can sometimes be seen below the corium. The outer limit of the pouch is formed by a layer of mesothelium.

Only one female was studied for changes immediately following ovulation without mating, but in this animal the most noticeable difference between her pouch and pouches observed before ovulation was in the mucous glands. The lumina of these glands tended to be occluded by the enlarged secretory cells (Fig. 8), in contrast to their condition before ovulation. No change was detected in the number of glands. Folds were conspicuous in the epithelium.

Ovulation is usually followed by mating and filling of the pouch with eggs. The initial changes associated with incubation of the eggs appear to be the development of activity in the mucous glands and the thinning of the pouch. Enlargement of the pouch seems to be the result of the presence of eggs, presumably due to

FIGURE 6. Living embryo of an advanced stage removed from the pouch and the jelly capsule. Before the jelly was removed, the gills completely enveloped the embryo, but removal resulted in collapse and shrinkage of the gills. The blood vessels within the gill stalks are prominent. Bar represents 2 mm.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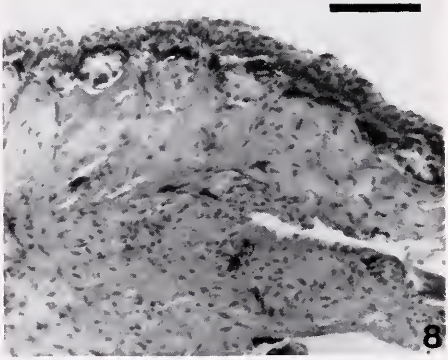
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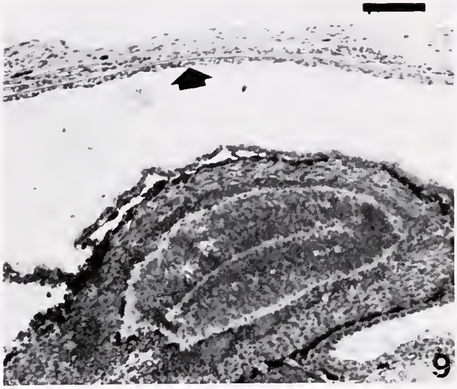
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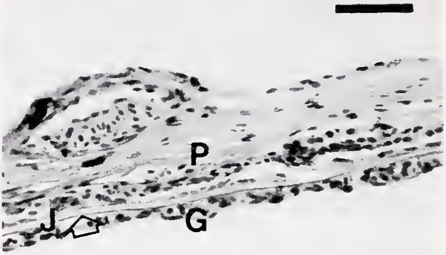

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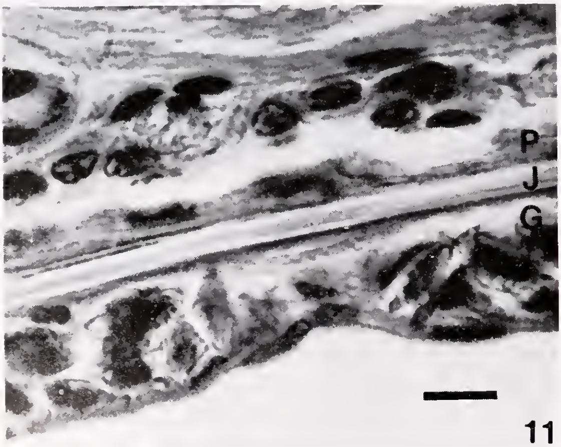

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FIGURE 7. Cross section of the pouch before ovulation. Infoldings of the epithelium are prominent, and there are numerous simple alveloar glands with empty lumina. The epithelial layer is less keratinized than the integument, but the general appearance is similar. Bar represents 100 μ.

mechanical distension, since we observed that in one female that oviposited but did not mate, the pouch remained the same size fifteen days after egg laying.

Embryonic development in the pouch is accompanied by increased vascularization of the pouch lining and close association between pouch and egg jelly (Figs. 9, 10 and 11). As the pouch walls become thinner, numerous blood vessels invade the area of the basal membrane and therefore come to lie very close to the epithelium (Figs. 10 and 11). The mucous glands have large lumina and are rare, compared to the previous condition. Initially, the walls of the pouch are in simple contact with the jelly capsules of the embryos, but as development proceeds, each encapsulated embryo comes to be partially enclosed in a vascularized pocket of pouch tissue. The chambers are formed by the upper and lower walls of the pouch and by lateral projections from these walls around the embryos.

Embryonic incubation in the pouch ends with hatching and release of the tadpoles. Birth of the tadpoles is followed by shrinkage and thickening of the lining of the pouch. Immediately after birth, the egg pockets of the pouch are still present but are collapsed (Fig. 12); the pouch appears disorganized when compared with that of the pregnant female.

Regression of capillaries in the pouch follows the birth of the tadpoles and the projections which formed the embryonic chambers are withdrawn. Epithelial folds are prominent immediately following birth (Fig. 12), but reorganization of pouch tissue eliminates them either by invagination of the epithelium, followed by tissue reorganization within the layer of connective tissue, or by evagination and shedding. Extensive shedding of the epithelium was noticed some days after birth of the tadpoles. During reorganization, numerous compound alveolar glands with large lumina appear, located deep in the layer of connective tissue (Fig. 13).

In a female examined fifty-two days after birth of the tadpoles, the structure of the pouch appeared similar to that of the sexually mature female before ovulation, but epidermal folds were inconspicuous. Mucous glands were present but were of the simple alveolar type, with large lumina.

Embryonic incubation and birth of tadpoles

Early development, occurring inside the pouch, is essentially synchronous. Development is associated with an increase in size of the embryos, and the pouch becomes much expanded as development proceeds. Segmenting eggs taken from the pouch are approximately 3 mm in diameter and are covered by a thin but firm coat of jelly; they lack dark pigment and the yolk gives them a rather uniform

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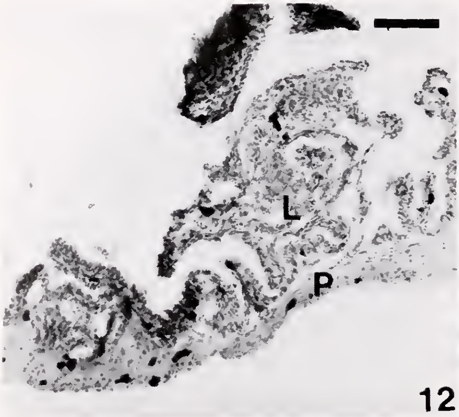
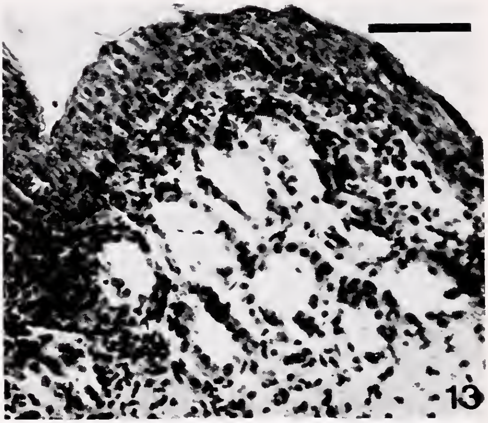
FIGURE 8. Cross section of the pouch immediately after ovulation. No eggs had been placed in the pouch. The pouch is similar to that prior to ovulation except for the mucous glands, whose cells appear to be enlarged with accumulated secretory material. Bar represents 100 μ.

FIGURE 9. Cross section of an embryo within the pouch. The embryo is surrounded by the fluid-filled space of the capsule. The bell gill (arrow) is flattened against the jelly, and the jelly lies against the vascularized tissue of the pouch. Detail is shown in the next two figures. Bar represents 100 μ.

FIGURE 10. Detail of association between bell gill (G), jelly (J), and pouch (P). Bar represents 50 μ.

FIGURE 11. Higher magnification of the gill-jelly-pouch relationship shown in Figure 10. Bar represents 10 μ.





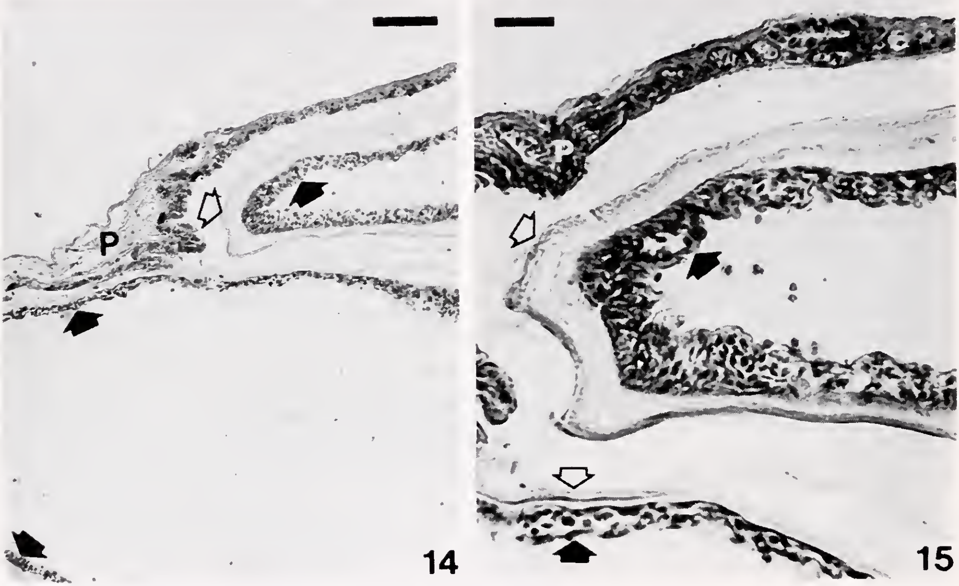


Figure 12. Pouch immediately following hatching and emergence of the tadpoles. The extensive folding and vascularization of the lining is evident. Bar represents 100 μ.

Figure 13. Reorganizing pouch 27 days after emergence of the tadpoles. Note the presence of a compound alveolar gland. Bar represents 100 μ.

Figures 14 and 15. Cross section of two adjacent embryos within the pouch. Each embryo is associated with the epithelium of the pouch, but the jelly capsules of the two embryos were probably contiguous with each other before shrinkage was induced by fixation. P represents pouch; open arrows, jelly; solid arrows, gill. The bar in Figure 14 represents 100 μ; bar, Figure 15, represents 50 μ.

yellowish-white color. The first cleavage divisions appear to divide the egg into four large blastomeres, but it is not known whether the cleavage furrows in the vegetal area are more than superficial. Development occurs at the animal end of the egg in a manner, described by Spannhof and Spannhof (1972), as similar to that of a fish. The early development of *Gastrotheca riobambae* in the pouch is indeed different from that described for anurans such as *Xenopus laevis* or *Rana pipiens* (Nieuwkoop and Faber, 1967; Rugh, 1962). A unique developmental characteristic of *Gastrotheca* is the early appearance and subsequent marked growth of a pair of expanded gills, called "bell gills" (Noble, 1927). These gills originate from two paired masses of tissue, the gill primordia, located on either side of the head of the early embryo (Fig. 4). The functional gills of *Gastrotheca* originate from the fusion of these two primordia on each side. In the early gills the area of fusion of the two primordia, indicating their double origin, is easily distinguished. Later, the gills become small vascularized discs; still later, when fully developed (Figs. 5 and 6), they completely surround the embryo, with the right and left gills joining to form a highly vascular sac in intimate contact with the inner surface of the jelly coat (Figs. 9, 10, and 11). Each gill is connected to the embryo-proper by two long vascular stalks (Fig. 6); each stalk consists of one efferent vessel and one afferent vessel within a common membranous covering. Therefore, between each gill and the embryo there are four connecting blood vessels. Later in development, the gills appear to cease growth, thus at the time of hatching and birth, since the embryo has continued to grow, the gills no longer envelop the entire embryo. Although the gills appear simply to halt their growth, it is possible that they may actually decrease in size toward the later stages of development. At hatching, the vascularized discs of both gills protrude through the left operculum, but the gill stalks have been retracted and are no longer visible externally. Immediately after birth the gills are bright red; after a few minutes of contact with pond water, however, the red color disappears. Apparently the circulation through the gills stops soon after hatching, after which gill resorption occurs, taking approximately 24 hours. The histological constitution of the fully developed bell gill (Figs. 9, 10, 11, 14, and 15) appears as a thin epithelium containing numerous blood vessels associated with the basal membrane. The gills bear a general resemblance to lung tissue.

During the initial stages of embryonic development there is minimal association between pouch and embryos; young embryos can readily be extruded to the outside by slight external pressure on the pouch. As development continues, howver, removal of embryos becomes somewhat more difficult. The increased difficulty of removal is due to the development of an intimate association between the gills of the embryo and the highly vascular lining of the pouch, with only the thin layer of jelly lying between the maternal and embryonic circulation (Figs. 9, 10, and 11).

Embryos may be distributed in the pouch in either a single or a double layer. In all cases, however, each embryo is in contact with pouch lining. Ordinarily, embryos do not seem to be completely enveloped by pouch tissue, although this might conceivably occur when there are few embryos in the pouch. Each embryo lies in an individual concavity of pouch tissue and in close contact with it, but the jelly of some embryos is contiguous (Figs. 14 and 15). In this situation, therefore, the vascular systems of adjacent embryos are separated from each other by their thin jelly capsules only.

The period of incubation within the pouch is somewhat variable. Under laboratory conditions we have observed a range of 103 to 120 days. Hatching usually occurs in the pouch, followed by emergence aided by the mother. Just before emergence of the tadpoles, the female moves into water and rests her forelegs on a supporting surface. She then inserts the long toes of her hind legs into the pouch and, after a few seconds, one or two swimming tadpoles emerge. The female usually remains motionless for less than a minute before repeating the insertion process. In the laboratory, on occasion, not all the tadpoles from a female hatched on the same day, but rather over a period of two or more days.

The number of embryos per pouch varies considerably: in 11 females, the number ranged from 81 to 207, with a mean of 125 ± 10 (s.e.). Unfertilized eggs or dead embryos in the pouch were rare, but when present, they had dried in the pouch and were expelled with the newly hatched tadpoles at the time of birth. Measurements of 42 newly hatched tadpoles from 8 females gave a snout to vent length of 7.7 ± 0.2 mm, with a total length of 20.2 ± 0.4 mm.

Segmenting eggs and very early embryos within the jelly do not survive in pond water but can be cultured in Ringer's solution for as long as seventeen days. Embryos cultured in Ringer's solution without aeration develop more slowly than those left in the pouch and appear to be deficient in blood formation. Aeration of the solution increases developmental rate and seems to improve blood production. Escape from the jelly does not occur in segmenting eggs and early embryos cultured *in vitro,* but advanced embryos hatch soon after being placed in pond water or Ringer's. Pouch embryos that have acquired the tadpole shape and have developed complete bell gills (Fig. 6) can be cultured either in pond water or Ringer's. In either medium, they soon hatch from the jelly and appear as very small free-living tadpoles. Resorption of the bell gills usually takes several days under these conditions.

As mentioned earlier, circulation thorough the bell gills apparently ceases soon after normal hatching, after which the gills are resorbed. If hatched tadpoles are kept in 1.5× Ringer's solution, however, the circulation and gills are maintained. Free-living tadpoles can be equally well maintained in pond water or in Ringer's solution.

DISCUSSION

Reproductive adaptations toward a terrestrial mode of life have evolved in a number of amphibian groups. Among the anurans, *Nectophrynoides occidentalis* shows true viviparity (Angel and Lamotte, 1948). Others have not gone that far, but have abbreviated the aquatic dependence for embryonic development in many different ways (*cf.* Gallien, 1959; Noble, 1931). Members of the genus *Gastrotheca* are examples of the latter group. In this genus the requirement of an aqueous environment for embryonic development has been diminished or essentially eliminated, depending on the species. In *Gastrotheca ovifera,* young metamorphosed froglets are born from the pouch, while in *Gastrotheca marsupiata* and *Gastrotheca riobambae,* tadpoles are born (Matthews, 1957; Hoogmoed, 1967; Spannhof and Spannhof, 1972; this paper). In this latter case, the tadpoles metamorphose after only a few weeks of aquatic development. Mating in *Gastrotheca* occurs on land, in contrast to the more usual situation for anurans.

In evolution, the pouch of *Gastrotheca* may have originated as a fold of dorsal skin serving to protect the embryos in a terrestrial environment. One may suppose that the pouch could have served originally as a structure protecting the embryos against predation; at the same time, the surrounding tissue would also insure the embryos against desiccation. It may be that the pouch of *Gastrotheca* originated as shallow folds of skin similar to those bordering the egg mass carried on the back of the female *Fritziana* (see Trueb, 1974, for a discussion of the possible phyletic relationship of the South American pouched frogs). Development of the pouch (Jones *et al.*, 1973), which involves invagination of the integument, is in agreement with that idea.

Noble (1925) found that, in the Amphibia, reduction of functioning of the lungs is correlated with modifications of the skin which make it more effective in respiration. One of the modifications involves the penetration of capillaries from the dermis into a closer association with the epidermis. According to Noble, these capillaries lie just beneath the outermost epidermal layer. In a similar way, the vascularization lying close to the epithelium of the pouch in *Gastrotheca* during pregnancy, together with the general thinning of the pouch wall, appear to transform the pouch of pregnant females into an organ functioning in gaseous exchanges between mother and embryos. Noble (1925) mentioned the pouch as a specialized respiratory organ, and Spannhof and Spannhof (1972) observed not only the vascularization of the pouch in *G. marsupiata* but also the formation of egg chambers. We have seen what is apparently a similar situation in *G. riobambae*. It seems evident that the vascularization of the outgrowths forming the egg chambers enhances the capacity of the pouch for gaseous exchange. The maternal and embryonic circulatory systems are separated only by the thin (about 10 μ) jelly capsule of the embryo, and this may be assumed to be of little hindrance to diffusion of gases.

In addition to the rather obvious respiratory function of the pouch, it is also possible that maternal-embryonic exchanges of water and/or other materials may occur between the closely apposed circulatory systems. Jones *et al.* (1973) support the idea of maternal-embryonic interchanges, but without reference to the nature of the materials supposedly exchanged and without specific evidence. Spannhof and Spannhof (1972), on the other hand, believe that probably no nutrient transfer occurs in the pouch, since they were able to maintain embryos and follow development for as long as 8 days outside the pouch. Evidence from the present study confirms the fact that embryos can be cultured outside the pouch for extended periods under certain conditions, but is of no help with regard to the matter of maternal-embryonic exchanges. Preliminary studies in our laboratory, however, reveal no appreciable change in the dry weight of embryos while in the pouch, suggesting that there is not a passage of nutrients from the mother into the embryo.

Hormonal factors are evidently important in pouch formation and function. Pouch formation has already been shown to be elicited by injection of estradiol into immature females (Jones *et al.*, 1973). Our observations suggest a hormonal role in opening and closing of the pouch aperture. It is possible that embryonic incubation, together with vascularization of the pouch and the formation of egg chambers, may also be under hormonal control. Preliminary experiments in our laboratory, using ovariectomized pregnant females, give support to this possibility. Pouch function, at least insofar as opening and closing of the aperture is concerned, seems also to be subject to nervous control. This is manifest in the rapid opening of the pouch aperture when a pregnant female is handled.

The embryonic bell gills of *Gastrotheca,* along with the pouch, are clearly important adaptations in terrestrial reproduction, and it is worth noting that most frogs with incubating pouches develop this type of gill (Noble, 1931). The evidence of the present study indicates a double origin of the gills, as does the work of Spannhof and Spannhof (1972), who traced the origin of the blood vessels of the gills of *Gastrotheca marsupiata* to the first and second aortic arches. The gills of *Gastrotheca* might have been multiple originally, similar to the situation in some species of *Cryptobatrachus* (Noble, 1927).

The study of reproduction in *Gastrotheca* is of considerable interest for comparative physiology, development and anatomy, as well as for the general problem of evolutionary adaptations to the terrestrial environment. In this case, the pouch and the pattern of development, along with associated hormonal and nervous control systems constitute remarkable modifications which make possible an extended period of independence from the aquatic environment. Further study of the details of reproduction in this species is in progress.

SUMMARY

The pouch of *Gastrotheca riobambae* (Fowler) serves as the location for development of the embryo up to the swimming tadpole stage. The pouch lies under the dorsal integument of the female and, in nonpregnant females, is similar to the integument. Pregnancy is accompanied by increased vascularization of the pouch. Blood capillaries from the corium become closely associated with the epithelial lining of the pouch, and vascularized outgrowths of the pouch partially envelop the embryos. Embryonic development in the pouch is characterized by the presence of peculiar gills, the highly vascularized "bell gills," which expand and flatten against the inner surface of the jelly capsule, forming an individual sac about each embryo and thus establishing a close relationship between embryonic and maternal circulatory systems. After birth of the tadpoles, the gills are soon resorbed and there is regression of the vascularization of the pouch. Reorganization of the tissue and shedding of the lining epithelium restore the pouch to the condition found in the nonpregnant female.

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SUMMER ABUNDANCE AND ECOLOGY OF ZOOPLANKTON IN THE GULF STREAM

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The Gulf Stream is an important ocean current that has been studied intermittently hydrologically and biologically. The hydrology includes the causes and theories of the Gulf Stream flow and meanders, all of which have been well summarized by Stommel (1958). Biological transects across the northern portion of the Gulf Stream (south of the Grand Banks) have been made by Clarke (1940) and Grice and Hart (1962), with the objective zooplankton description on either side as well as in the Gulf Stream.

However, it is of interest to trace continuously the zooplankton population density in a volume of water as it drifts north in the Gulf Stream flow for a considerable portion, attempt to correlate it with the hydrology, and hopefully arrive at some ecological relationships. These relationships would include the incident solar radiation, primary production, grazing by zooplankton, effects of surface winds and Gulf Stream eddy viscosity on mixing and water transport, and effect of dissolved minerals on zooplankton population. This is indeed a formidable objective and would require a very extensive observational program to accomplish completely; fortunately, we were able to acquire data for this analysis in July–August 1969 from the USNS Lynch (T-AGOR 7) and a manned submersible, the Ben Franklin (PX-15). The drift of the Ben Franklin was closely followed with the locations of 42 horizontal tow samples taken from the Lynch (Egan, 1974).

The three zones of water between our eastern coast of the United States and Bermuda are of great biological interest because of their extreme contrast (Clarke, 1940), in particular the Gulf Stream and the slope water, which is the area of mixing of the continental slope water with the Gulf Stream. The Sargasso Sea bounds the eastern and southern boundaries of the Gulf Stream. Clarke's (1940) sampling program consisted of oblique hauls (one shallow and one deep) at nine stations along a transect south of the Grand Banks. Grice and Hart (1962) also made oblique tows in the same general area.

However, our hauls were horizontal at various depths because we sought to determine whether there was a variation in zooplankton populations with depth and location in the Gulf Stream. A single oblique tow throughout the water column at each station would have yielded far more reliable data on zooplankton abundance and biomass, but no information on the depth distribution. The depth distribution of zooplankton in the Gulf Stream is an important parameter, and the sampling program was planned to follow the course of the free drift of the Ben Franklin in the Gulf Stream. Thus the same region of the Gulf Stream would be sampled as it flowed north. During the free drift north of the Ben Franklin, optical measurements of the chlorophyll-*a* and chlorophyll degradation products were also made as a function of depth (Egan, 1974). It would then be possible to seek a relationship between these determinations and the depth distribution of zooplankton. Because of the low zooplankton population density, long duration tows were required. Also, for measurements of phytoplankton population, as indicated by the amount of chlorophyll-*a*, a very sensitive fluorescence measurement technique was required because of the low level of chlorophyll-*a* i.e., less than 1 $\mu g/l$ in the open ocean (Yentsch and Menzel, 1963).

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The hydrology of the Gulf Stream becomes important when we consider the greatly increased volume of water transport as the Gulf Stream expands from Cape Kennedy northward. This requires mixing with adjacent slope waters, concomitant with surface mixing caused by winds and tides. The transport at 30°30′N (off Jacksonville, Florida) is 37.2×10^6 cubic meters/second and at 32°30′ N (off Charleston, South Carolina), it is 53.0×10^6 cubic meters/second (Richardson, Schmitz, and Niiler, 1969). Further, a compilation of data by Knauss (1969) indicates the transport at 34°45′ N (off Cape Hatteras, North Carolina) to be 60×10^6 cubic meters/second (Barrett, 1965), and at 37°35′ N 65° E (south of Nova Scotia) to be 147×10^6 cubic meters/second (Fuglister, 1963). Coastal oceanographic and sedimentologic interpretation of Apollo IX space photographs show plume like patterns of sediment bounding the western edge of the Gulf Stream (Mairs, 1970). It would be assumed that some of these sediments with some of the biota in these coastal waters enter the Gulf Stream. Further, the lateral meanders and detached eddies of the Gulf Stream between Cape Hatteras and the Grand Banks (Hansen, 1970) would also cause mixing with adjacent waters, together with momentum transfer with the resulting observed water-transport increase (Knauss, 1969).

Tides would not be expected to cause any appreciable mixing effect, but would cause divergences: *i.e.,* a westward tide in the cooler Sargasso Sea would result in this water flowing down and under the Gulf Stream. Mixing with the Gulf Stream could occur by eddy turbulence along the edge irregularities (von Arx, Bumpus, and Richardson, 1955), caused by turbulent shearing stresses, as yet poorly elucidated (Stommel, 1958).

Minerals picked up by the Florida Current in the Gulf of Mexico and Caribbean Sea (Jacobs and Ewing, 1969) are transported into the Gulf Stream, possibly providing nutrients for primary production.

Primary production in the Gulf Stream is nutrient- or micronutrient-limited, since there is low attenuation by the sea water of incident solar irradiance. The amount of primary production in the Gulf Stream may be inferred from a measurement of chlorophyll-*a* and chlorophyll degradation products. This primary production in turn will support the Gulf Stream zooplankton populations. An ecological analysis could utilize previously published Gulf Stream measurements of cholorophyll-*a*, chlorophyll degradation products, and dissolved minerals.

TABLE I

Haul data and results.

Haul Stations	Latitude, °N		Longitude, °W		Depth of Tow, m	Duration of Tow (min)	Time Begin Tow (Z)	Surface Temperature °C	Date (1969)	Major Taxa Present	Ratio of Zooplankton to Phytoplankton
	Begin	End	Begin	End							
1	29°06.2′	29°08′	79°51.2′	79°51.1′	250	34	2242	30.1	18/7	16	3.0
2	29°32.2′	29°34′	79°50.1′	79°51′	306	30	1428	30.1	19/7	17	1.0
3	"	"	"	"	322	30	1428	30.1	19/7	14	0.97
4	29°50.3′	29°52.5′	80°00.6′	80°00.5′	310	60	2217	29.9	19/7	18	5.1
5	30°15.9′	30°18′	79°56.8′	79°58.0′	89	30	1530	29.9	20/7	16	8.0
6	30°36′	30°40′	80°03.6′	80°06.0′	25.7	60	2319	29.3	20/7	16	3.5
7	"	"	"	"	141	60	2319	29.3	20/7	20	10.7
8	31°22.0′	31°25.8′	79°35.0′	79°35.6′	22.6	60	2000	29.6	21/7	10	1.2
9	"	"	"	"	224	60	2000	29.6	21/7	20	2.3
10	"	"	"	"	1	60	2000	29.6	21/7	17	0.13
11	31°14.4′	31°16.6′	79°04.0′	79°03.0′	25.8	57	0051	29.4	22/7	19	2.1
12	31°19.5′	31°21.6′	79°00.2′	78°59.6′	23.7	56	0245	29.3	22/7	15	1.3
13	32°05.2′	32°06′	77°41.5′	77°39.5′	102	30	1830	28.9	24/7	12	2.1
14	32°19′	32°19.5′	77°33.5′	77°33.0′	92.8	30	0411	28.6	25/7	14	0.88
15	"	"	"	"	243.5	30	0411	28.6	25/7	16	7.6
16	"	"	"	"	1	30	0411	28.6	25/7	20	0.27
17	32°51.2′	32°53.8′	76°29.0′	76°27.4′	14.1	32	0045	29.4	27/7	18	1.0
18	"	"	"	"	95	32	0045	29.4	27/7	15	0.71
19	"	"	"	"	3	32	0045	29.4	27/7	19	0.27
20	32°54′	32°55.4′	76°24.0′	76°23.0′	29.5	30	0422	29.4	28/7	19	0.31
21	"	"	"	"	65	30	0422	29.4	28/7	19	0.75
22	33°36.0′	33°38.0′	76°13.5′	76°11′	19	60	0147	28.7	29/7	18	0.61
23	"	"	"	"	79.5	60	0147	28.7	29/7	17	0.80
24	35°12.6′	35°14.0′	74°00.5′	73°58.3′	38	60	1806	28.2	1/8	16	0.71
25	"	"	"	"	100	60	1806	28.2	1/8	6	0.46
26	35°35.0′	35°35.7′	74°12′	74°15.4′	21.8	60	2345	27.8	1/8	12	0.61
27	"	"	"	"	288	60	2345	27.8	1/8	17	6.4
28	35°33.4′	35°35.0′	74°00.0′	73°58.0′	81.8	60	0215	27.8	2/8	13	0.89
29	"	"	"	"	384	60	0215	27.8	2/8	15	2.8
30	37°07′	37°11′	70°20′	70°17′	70	60	0045	28.2	5/8	16	0.21
31	"	"	"	"	318	60	0045	28.2	5/8	12	1.1
32	37°22.0′	37°26.0′	70°13.0′	70°09′	187	60	0325	27.2	5/8	16	6.0
33	"	"	"	"	201	60	0325	27.2	5/8	17	1.9
34	37°05.0′	37°07.0′	69°44.5′	60°39′	37.4	60	1030	27.4	5/8	17	0.76
35	"	"	"	"	60	60	1030	27.4	5/8	18	1.7
36	37°43′	37°39.9′	62°23′	69°18.0′	378	60	0627	27.8	6/8	15	5.5
37	"	"	"	"	386	60	0627	27.8	6/8	15	2.3
38	37°41′	37°47′	69°17′	69°13′	547	60	0837	27.8	6/8	13	3.3
39	38°25′	38°24′	66°28′	66°26′	110	60	0220	24.8	8/8	16	3.9
40	"	"	"	"	48	60	0220	24.8	8/8	9	0.20
41	37°48′	37°51′	62°39′	62°37′	233	60	2015	27.5	11/8	16	2.0
42	"	"	"	"	466	60	2015	27.5	11/8	16	2.6

MATERIALS AND METHODS

There are two aspects of the experimental procedure for the determination of the summer abundance of zooplankton in the Gulf Stream. The first involves the collection program from the Lynch cruise, and the second involves the classification and population determinations in the laboratory.

Forty-two horizontal plankton hauls from the Lynch were made at 23 locations listed in Table I. Depths ranged from 1 to 547 meters and the durations were usually 60 minutes, with some shorter, at varying times of day and night (Table I); the surface water temperature was indicative of the Gulf Stream water at all stations on the dates shown. A number of hauls were made at nearly the same depths (Station 6, 8, 11, 12, and 26; 36 and 37; 32 and 33; 10 and 16) to check the consistency between tows.

Tows were made with a 12-inch (30.5 cm) mouth Clarke-Bumpus automatic plankton sampler with a 100 μm mesh (Kahl Scientific Instrument Corporation). The counter on the flow meter was later found to register improperly, and the flow was estimated based on published data. The Clarke-Bumpus towing assembly is unflared. Tranter and Heron (1967) give the filtration efficiency as 80% at a towing speed of 1 knot with a mesh width of 260 μm. Our towing speed was 1 knot for all samples and the mesh opening was 100 μm. Using Table 9 of Tranter and Heron (1967) and interpolating for a 100 μm mesh opening, the effect of this opening compared to a 260 μm mesh is an efficiency of 88%. The over-all efficiency is the product which is 70.4%. The net had a porosity of 36%. The volume of water passing through this net is reduced below that passing through the 30.5 cm diameter mouth of the sampler by the efficiency of the sampler and the porosity of the net. The volume of water passing through this net in 1 hour at a 1 knot towing speed is 34.2 cubic meters. Tranter, Kerr, and Heron (1968) indicate that a 1 knot hauling speed is reasonable.

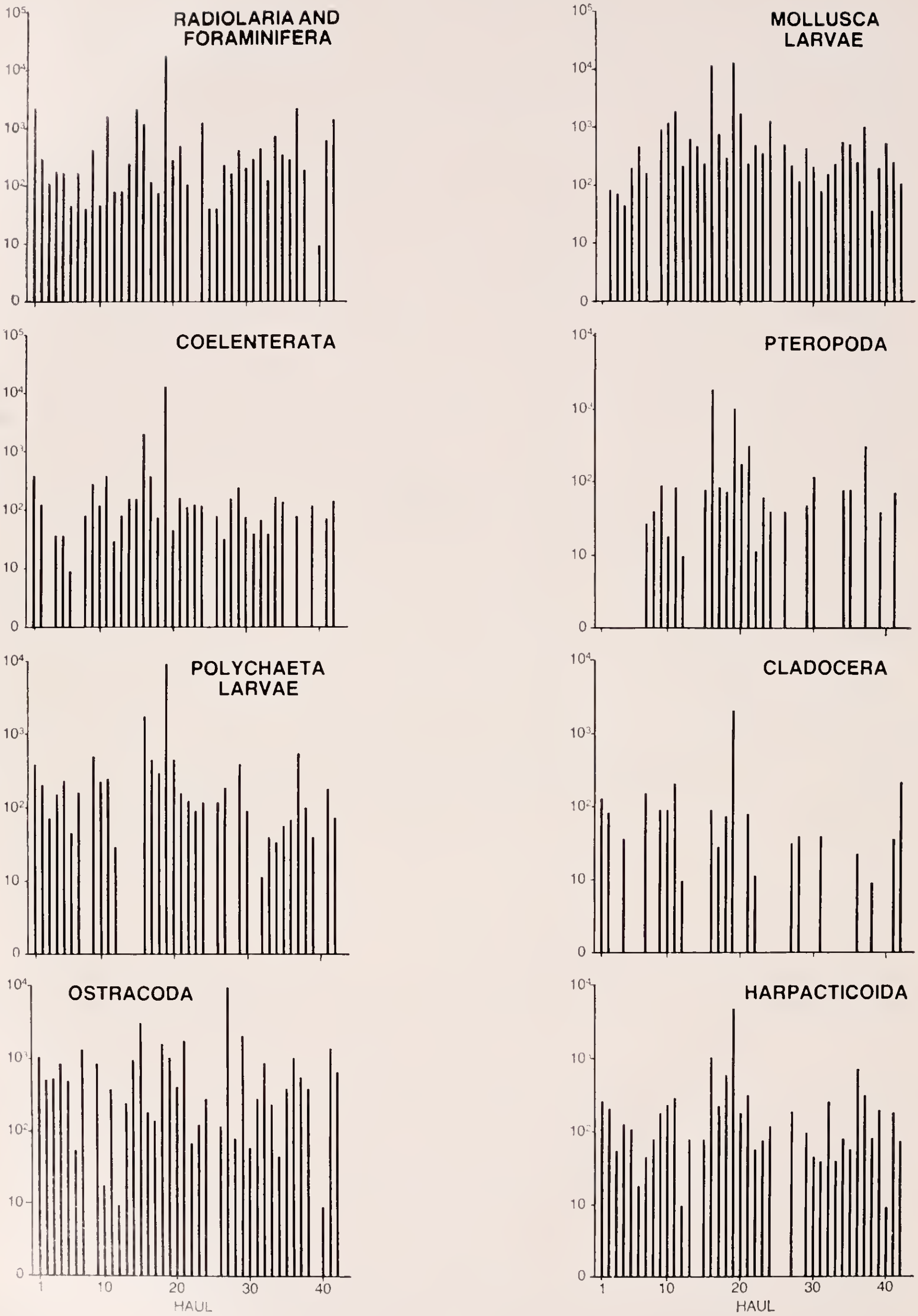
The jumbo Clarke-Bumpus sampler is of reasonable size for depths up to 500 meters, being comparable with the 80 cm diameter Juday nets used by Soviet expeditions for these depths (Vinogradov, 1970); their mesh size was 180 μm, and Riley (1939) for instance, used a 160 μm mesh. Our use of a 100 μm mesh net was intended to capture smaller as well as large zooplankton. The mesh was also fine enough to capture large phytoplankton and yet not clog with nannoplankton. The Gulf Stream nevertheless generally has a low phytoplankton population (see Egan, 1974, for instance). Tows were made at varying times of day.

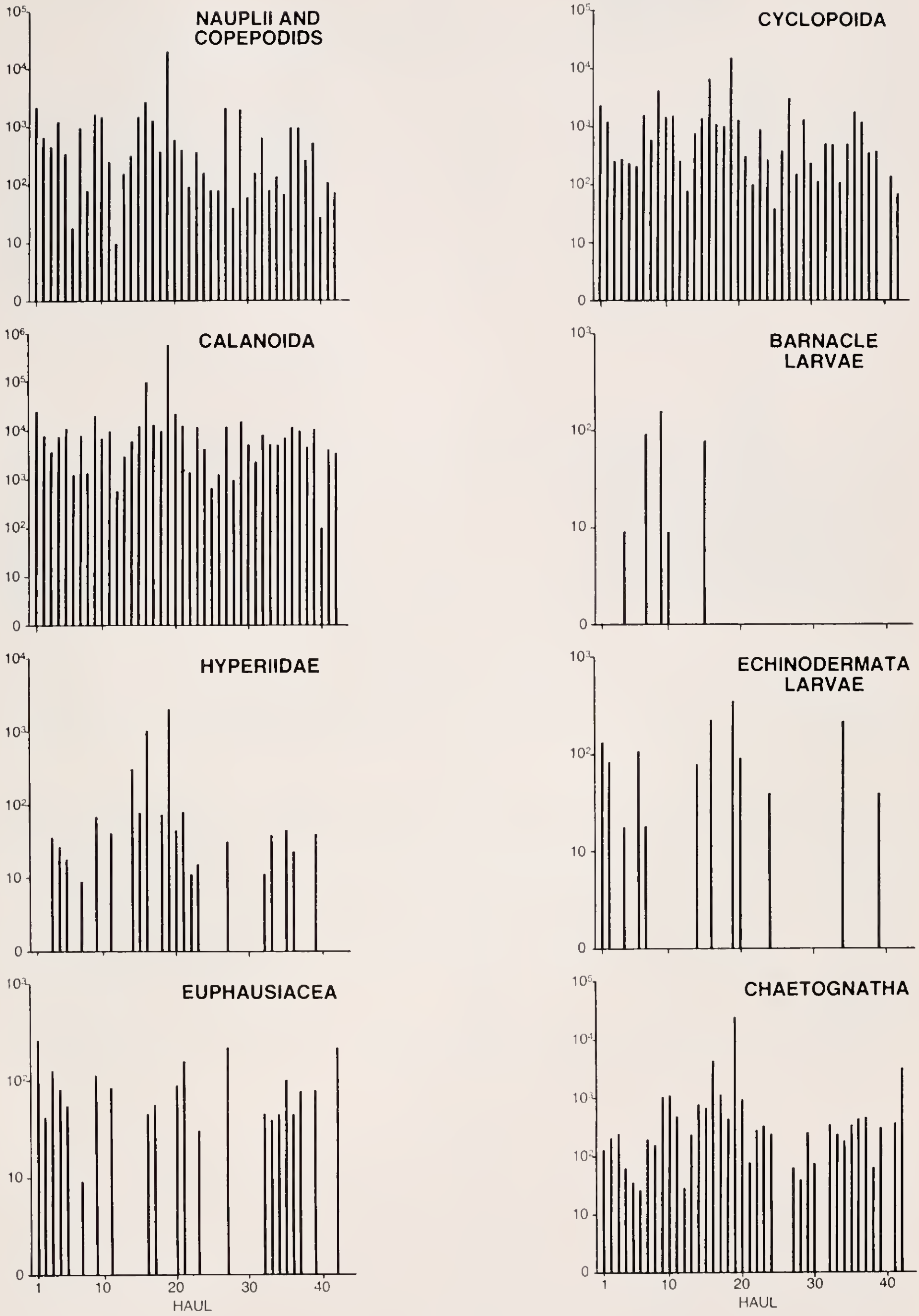
Concurrently with the plankton sampling, observations were made of sea conditions, surface winds, and cloud cover; the object of these observations was to infer the degree of sea surface mixing in the Ekman wind-drift layer (Stommel, 1958) and to determine the solar irradiance available for primary production.

Collected zooplankton were preserved with a formalin solution and stored in Mason jars. Separation of the zooplankton was accomplished by allowing the contents of the Mason jars to settle, which took four years because of the priority of validating our optical instrumentation (Egan and Cassin, 1973). A measured portion of the supernatent liquid in the jars was decanted (about half) because it contained little or no biota and the remainder was a concentrate of zooplankton. It was stirred to produce a homogeneous mixture. Between 4 and 10 four-milliliter aliquots were pipetted off this concentrated mixture and analyzed. The number of aliquots depended upon the density and diversity of the sample. The data was corrected to the original full sample volume.

Using a gridded counting disk, the total number of zooplankton in each aliquot was counted with a binocular stereo microscope at magnifications up to 70×. Additional identifications were made with a compound microscope at magnifications up to 1000×. Ratios between phytoplankton retained in the net and zooplankton were calculated. The number of phytoplankton was determined by counting large cells (*e.g., Ceratium*) and chains of smaller cells. Thus the ratio was not calculated as cells per zooplankter, but alternately as large cells and chains per zooplankter. Four replicates were counted for each sample.

In order to get an estimate of the zooplankton biomass, dry weight determinations were made. A correct determination would have required destruction of all our samples, which still could be useful for species determinations beyond that reported on in this paper. In order to conserve the major portion of our samples, we adopted an approximate approach. The method consisted of counting out


RADIOLARIA AND FORAMINIFERA
MOLLUSCA LARVAE
COELENTERATA
PTEROPODA
POLYCHAETA LARVAE
CLADOCERA
OSTRACODA
HARPACTICOIDA
10^5
10^4
10^3
10^2
10
0
1
10
20
30
40
HAUL


NAUPLII AND COPEPODIDS
10^5
10^4
10^3
10^2
10
0
CYCLOPOIDA
10^5
10^4
10^3
10^2
10
0
CALANOIDA
10^6
10^5
10^4
10^3
10^2
10
0
BARNACLE LARVAE
10^3
10^2
10
0
HYPERIIDAE
10^4
10^3
10^2
10
0
ECHINODERMATA LARVAE
10^3
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0
EUPHAUSIACEA
10^3
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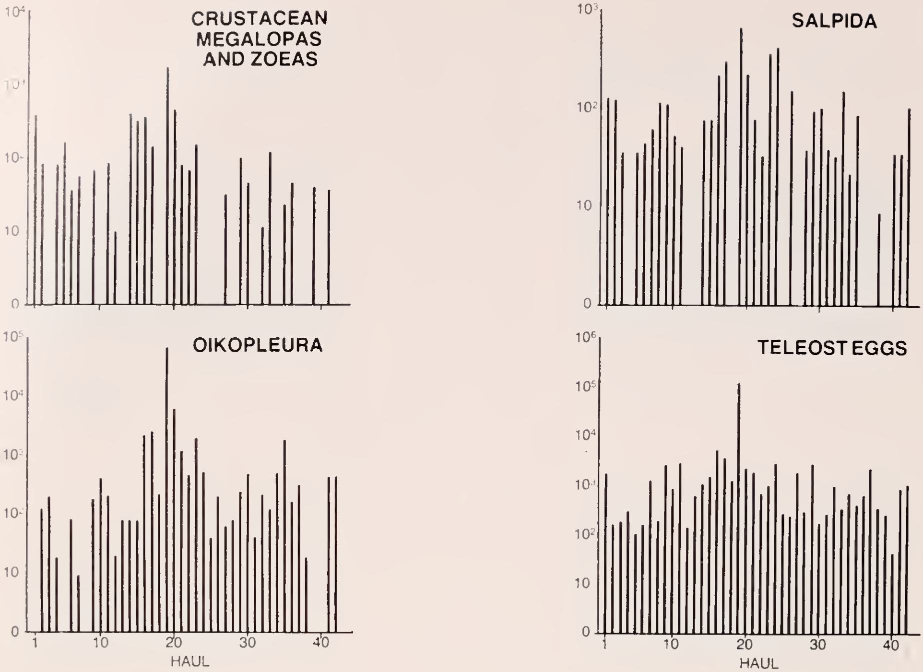

FIGURE 1. Comparison of zooplankton populations by taxa and haul. Vertical axes represent individuals per 100 m³, and the horizontal axis represents the 42 hauls in chronological order. (Data on file at National Oceanographic Data Center Accession Number 75–0732.)

the first 500 zooplankton individuals and filtering them on a tared millipore filter pad that had been previously heated for one hour at 100° C. The sample was then placed in a drying oven at 100° C for a period of 24 hours, after which time it was weighed on a Mettler H20 balance, and the weight recorded. Replicates from three random stations were made. Calculations could then be made of the average weight per zooplankton individual. This factor was then multiplied by the average number of individuals per m³ in order to arrive at the dry weight biomass determination.

Correlation coefficients were calculated relating total zooplankton and taxa to depth, local time of day, latitude, and longitude.

RESULTS

Laboratory analyses of the 42 hauls are presented in Figure 1. There are twenty taxa represented, and the populations per 100 m³ are plotted versus station (the numerical data is on file at the National Oceanographic Data Center, Accession Number 75–0732). A logarithmic ordinate is used because of the large range of population variation between stations. A zero ordinate indicates absence of the taxon at the station. Additionally, Table I lists, by haul, the number of

TABLE II

Correlation matrix for total zooplankton with depth, location, and local time, and comparison with Gulf Stream chlorophyll data.

	Depth	Latitude	Longitude	Time
All zooplankton (Time: 0 to 1200 hr)	0.035	0.298	−0.267	−0.533
All zooplankton (Time: 1200 to 2400 hr)	−0.186	−0.102	0.090	0.054
Zooplankton at depths <100m	−0.329	−0.105	0.077	0.106
Zooplankton at depths between 100 and 300m	0.660	−0.552	0.555	−0.137
Zooplankton at depths >300m	0.007	0.221	−0.092	−0.055
Chlorophyll-*a* and chlorophyll degradation products	0.054*	0.211*	−0.317*	—

* Calculated from data of Egan, 1974.

major taxa present and the ratio of zooplankton to phytoplankton. Table II presents the correlation matrix for total zooplankton with depth, location (in terms of latitude and longitude), and local time, with a comparison to cholorphyll data. Table III lists the average population per 100 m^3 over all 23 stations and the depth and latitude correlations.

Referring to the taxa shown in Figure 1, and Table I (the next to the last column where the number of taxa present are listed as a function of depth), the correlation (ranges from +1 through 0 to −1, where 0 is no correlation) between

TABLE III

Average population per 100m^3 over all 42 hauls, and correlation coefficients for depths and latitude.

Taxa	Population	Depth Correlation	Latitude Correlation
Radiolaria and Foraminifera	833	−0.10	−0.07
Coelenterata	443	−0.19	−0.09
Polychaeta larvae	402	−0.18	−0.10
Mollusca larvae	1,000	−0.29	−0.12
Pteropoda	114	−0.24	−0.09
Cladocera	84	−0.14	−0.11
Ostracoda	794	0.29	0.02
Nauplii and Copepodids	1,079	−0.11	−0.13
Calanoida	23,590	−0.18	−0.09
Harpacticoida	275	−0.15	−0.07
Cyclopoida	1,321	−0.14	−0.18
Barnacle larvae	8	0.10	−0.25
Hyperidae	98	−0.23	−0.12
Euphausiacea	48	0.32	−0.10
Crustacean megalopas and zoeas	121	−0.20	−0.22
Echinodermata larvae	33	−0.27	−0.18
Chaetognatha	1,040	−0.16	−0.07
Salpida	100	−0.32	−0.11
Oikopleura	2,106	−0.19	−0.07
Teleost eggs	4,174	−0.17	−0.07
Miscellaneous	51	−0.10	0.05
Total	37,714	−0.18	−0.09

taxa and depth is small (calculated to be +0.037). Some regions of the ocean have a very strong dependence of species on depth (Vinogradov, 1970). However, there is a stronger correlation of the ratio of zooplankton to phytoplankton (last column, Table I) with depth (calculated to be +0.357).

If we consider the total zooplantkton population, and calculate the correlation matrix for depth, location, and local time, we obtain Table II. The matrix breaks up the time analysis into two intervals 0 to 1200 hr and 1200 to 1800 hr to check migration. The time is taken as the average time of the haul and corrected for local time at the tow site. There is a significant correlation with time between 0 and 1200 hr indicating a depletion of the zooplankton in the sampling depth range. Also a latitude-longitude dependence appears, the latitude effect being the opposite of longitude (resulting from the course of the cruise); the strongest effect is an increase in total zooplankton between 0 and 1200 hr with increasing latitude.

If we consider depth effects (Table II) and use three depth intervals (< 100m, 100 to 300m, and > 300m), we see that the total zooplankton decrease with depth for < 100m, increase with depth between 100 and 300m, and have negligible dependence at > 300m. The latitude dependence shows a depletion of the region < 300m depth with progress northward and an increase at depths > 300m.

A comparison with data derived from Egan (1974) indicates an increase in chlorophyll-*a* and chlorophyll degradation products with northward progress of the Gulf Stream. In comparison, the calculated correlation of the total zooplankton with latitude is negligible (−0.090).

Referring to Figure 1 and Table III, and based on more detailed correlation analyses by us, there is no unique correlation of the 20 taxa with depth, location, or time of day. Only latitude is given because of its approximately inverse relation to longitude for this cruise. The miscellaneous classification included such minor zooplankton as enteropneust larvae, teleosts, isopods, sponge amphiblastula, nematodes, *Ergasilus,* and phoronid actinotrocha. Also, all hauls included phytoplankton that did not pass through the 100 μm mesh plankton net; typical were *Ceratium, Chaetoceros, Asterionella,* pennate diatoms, and long unidentified chains. The numerical ratio of zooplankton to phytoplankton varied from 0.13 to 10.7, averaging 2.33 (Table I); however, this ratio is only a qualitative indication of the presence of phytoplankton because many long chains were observed, each counted as one, even though consisting of many cells. Also nannoplankton that passed through the net mesh would cause an additional phytoplankton volume that is not enumerated.

Three surface tows were made (Hauls 10, 16, and 19, Table I). Two show extraordinarily high populations, particularly calanoids (Hauls 16 and 19). These latter two hauls were off the coast of Georgia where unusually large standing crops may occur (Hart, 1942). These hauls evidenced large phytoplankton content also.

Even though Haul 38 was taken at a depth of 547 meters (Table I), there was a calanoid population of 2600 per 100 m^3 (Figure 1). Comparing this to the average over 42 hauls (Table III and Figure 1), it can be seen that the calanoid population may vary greatly from haul to haul with appreciable population at greater depths. Figure 1 also depicts that the ratio among the taxa between hauls may vary considerably.

Another interesting observation exists for Hauls 36 and 37; both were made at the same time, at almost the same depths (378 and 386m, Table I). The calanoid population for Hauls 36 and 37 was 11600 and 9860 per 100m^3 (Figure 1 and Table I), and the number of major taxa present was the same (15).

There are other comparisons that may be made among the data that show that the zooplankton population distribution and concentration varied considerably during July and August, 1969, in the Gulf Stream. However, as noted previously, there does not appear to be any consistent buildup in any of the zooplankton population taxa with northerly drift in the Gulf Stream. Nor does there appear to be any systematic decrease.

Discussion

The most significant result of this study is that there is no significant over-all population increase or decrease of zooplankton with progress north in the Gulf Stream under the summer conditions that existed in 1969. Also, there is no significant consistent correlation of total zooplankton with depth, time of day, or location.

In order to explain these seeming inconsistencies, we must consider the hydrology of the Gulf Stream, and concur in the hypothesis that there must be a considerable influx of zooplankton-laden continental shelf water (Warren and Volkmann, 1968). This influx of continental shelf water mixing with the Gulf Stream would be expected to cause considerable variations in the zooplankton (and phytoplankton, as well as nutrients) and result in the observed biological data.

It has already been noted that the volume transport of the Gulf Stream increases with northward progress. Preliminary analyses indicate that the mechanism of momentum transfer from the Gulf Stream is by means of a geostrophically-balanced inflow (Sturges, 1968); the deep water shear is about 1 cm/sec in 3 km. This geostrophically balanced inflow is not a meander that may grow into a detached eddy (Hansen, 1970). Surface mixing to the depth of the Ekman Layer (about 50m) can occur by means of shear from surface winds (Stommel, 1958).

The present concept of Gulf Stream transport deduced from the observations of Warren and Volkmann (1968) (between 37 to 38° N and 67 to 71° W) implies a considerable inflow of slope water. There is also a southward flow of Labrador Sea water (which could contain zooplankton) at depths between 1000 to 3000m underneath and into the Gulf Stream. The relative inflow amounts are conjectured to be 5×10^6 m^3/sec on the inshore side of the Gulf Stream and 30 or 40×10^6 m^3/sec on the offshore side (W. Sturges, Florida State University, Talahassee, private communication, 1974). It is conceded that a noncontroversial hydrological model of the Gulf Stream does not exist. There is no consistent trend of zooplankton population along the surface ship cruise that moved with the velocity of the Gulf Stream (*i.e.,* approximately 1.6 knots: Naval Oceanographic Office, 1965). A decrease in zooplankton population or increase should show some trend with drift northward in the Gulf Stream.

One might conjecture that the forementioned influx of Labrador Sea water would carry with it a representative boreal zooplankton population and nutrients. Boreal species are fewer than warmer water types (Sverdrup, Johnson, and

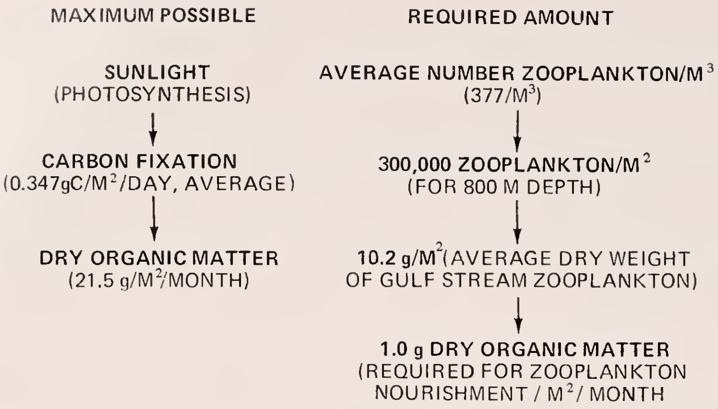
MAXIMUM POSSIBLE
REQUIRED AMOUNT
SUNLIGHT (PHOTOSYNTHESIS)
AVERAGE NUMBER ZOOPLANKTON/M³ (377/M³)
CARBON FIXATION (0.347gC/M²/DAY, AVERAGE)
300,000 ZOOPLANKTON/M² (FOR 800 M DEPTH)
DRY ORGANIC MATTER (21.5 g/M²/MONTH)
10.2 g/M² (AVERAGE DRY WEIGHT OF GULF STREAM ZOOPLANKTON)
1.0 g DRY ORGANIC MATTER (REQUIRED FOR ZOOPLANKTON NOURISHMENT / M² / MONTH

FIGURE 2. Gulf Stream ecology (zooplankton nourishment).

Fleming, 1942), but identification to the species level was not included in our observations. These determinations could be accomplished subsequently but are not essential to this paper.

As mentioned in the introduction, Clarke (1940) and Grice and Hart (1962) have studied the abundance of zooplankton along transects south of the Grand Banks. Also, Bowman (1971) has studied the distribution of calanoid copepods off the southeastern United States between Cape Hatteras and southern Florida. Clarke (1940) determined zooplankton abundance by means of a volume measurement and found (in agreement with us) that the amounts of plankton caught were extremely variable, even at neighboring stations.

Grice and Hart (1962) found in three collections that the zooplankter abundance in the Gulf Stream ranged between 99 and 156/m^3, which is less than our average of 377/m^3 (Table III).

Bowman (1971) quantitatively determined individual species of calanoids, and it is possible to extend our work by doing this; but we primarily sought to determine general classifications in order to analyze the ecology of the Gulf Stream and the additional detail was unnecessary.

The characterization of the Gulf Stream ecology is outlined in Figure 2. A comparison is made between the calculated dry organic matter available and the food requirements of zooplankton in the Gulf Stream. These calculations are based on the measurements described in this paper with that deduced from published experimental and theoretical relationships.

In detail, Table III indicates that there are on the average 377 individuals/m^3 for the cruise considered. The depth of the Gulf Stream is assumed to be approximately 800m (Naval Oceanographic Office, 1965). We have determined that the average dry weight of a Gulf Stream zooplankter is 0.034 mg, and in June about 10% of the dry zooplankton weight is required to support it for a month (Riley and Bumpus, 1946, based on Georges Bank data). Then 1.0 g/m^2 per month of phytoplankton would be required to nourish the zooplankton (Figure 2).

We compared this calculated requirement with that possible based on photosynthesis and measured cholorophyll concentrations in the Gulf Stream. Because the photosynthetic rate depends upon the incident solar irradiance, bi-hourly observations were made of cloud cover (C_0) during the cruise and this permitted average daily solar radiation (Q) totals to be calculated from Kimball, 1928; *i.e.*, $Q = Q_0\ (0.29 + 0.7\ [1.0 - C_0])$. The value of Q_0 was taken as 432 for July and 399 for August in gram cal/cm^2. The amount of carbon fixed per day was calculated from the expression $p = R/k \times C \times 3.7$ (Ryther, 1956). An average was then calculated over the cruise duration.

For clear (Gulf Stream) water, the extinction coefficient is $k = 0.02$. The factor R depends upon Q, and is determined from a graphical relation between solar radiation and relative photosynthesis rate (Ryther and Yentsch, 1957). The quantity C in grams of cholorophyll per cubic meter in the water column is based on *in situ* Gulf Stream measurements of cholorophyll (Egan, 1974) made concurrently with the net tows. Riley (1939) has made measurements of the vertical distribution of plant pigments in the Gulf Stream; the concentration approaches zero at a depth of 400m, with the average approximately given by the numerical value at 200m depth. [Even though Riley, 1939, used Harvey plant pigment units (HPPU) in the paper, there is a conversion relationship to chlorophyll given by Strickland, 1960: mg chlorophyll = $(3 \pm 1) \times 10^{-4}$ Number of HPPU. This value agrees with that calculated from (Riley, 1939) surface measurements within a factor of 2.09]. Riley (1939) thus indicated that the average level in the Gulf Stream would be about (0.3 ± 0.1) mg/m^3 of chlorophyll. The chlorophyll-*a* and cholorophyll degradation product measurements of Egan (1974) were made at depths from 120 to 569m, at an average depth of 253m. Using this data (average 0.14 mg/m^3 chlorophyll-*a* and chlorophyll degradation products) as representative of the average level of cholorophyll per cubic meter in the water column, we may then compute the quantity of carbon fixed per day, taking into account cloud cover. The average quantity of carbon fixed was calculated to be 0.347 g/m^2/day. The corresponding dry organic matter (17 μg carbon $\equiv$ 35 μg dry organic matter: Cushing, 1958) was 21.5 g/m^2/month (30 days). This is at least ten times that required to sustain the zooplankton population throughout the Gulf Stream (Figure 2) and would lead to a phytoplankton excess. This does not occur (Riley, 1939). It is rather improbable that there is a nutrient limitation because the nitrate to phosphate ratio was found to be 15:1 for the southern stations (Riley, 1939); Ryther and Dunston (1971) have shown that the inorganic nitrogen often appears to be the limiting factor in algae production in coastal waters. The southern station value of 15:1 is considerably higher than their value of 6:1. Various mineral sources, and transport of these minerals, have been reported that can supply the Florida Current, and subsequently the Gulf Stream (Jacobs and Ewing, 1969). It is possible that there is a micronutrient deficiency (such as iron, manganese, or vitamin B_{12}) which could limit the phytoplankton population (Raymont, 1963).

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SUMMARY

We have described the results of laboratory analyses of 42 horizontal tows using a Clarke-Bumpus plankton sampler with a 100 μm mesh net in the Gulf Stream. Classifications of 20 zooplankton taxa have been made for the 42 hauls. Calanoids dominate the hauls. It was found that the zooplankton population distribution and density varied considerably from haul to haul, but there was no consistent buildup or depletion of population with progress of the flow north in the Gulf Stream. These biological arguments reinforce previous conjectures that there is a geostrophically-balanced inflow of continental shelf and Labrador Sea water that gradually mixes with the Gulf Stream water mass. These inflows provide sources of zooplankton and phytoplankton to the Gulf Stream as it progresses northward. Also, an ecological analysis of the Gulf Stream indicates that the dry organic matter is over an order of magnitude below that which is theoretically possible.

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JUVENILE HORMONE TITERS IN THE HEMOLYMPH DURING LATE LARVAL DEVELOPMENT OF THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.)

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The juvenile hormone (JH), as with many other developmental hormones in insects, must reach its target tissues by way of the hemolymph. Thus, the hemolymph concentration bears an important relation to effects of JH (see de Wilde, de Kort and de Loof, 1971), although tissue retention of hormone or of "covert effects" of hormone may be of significance as well (*e.g.,* Ohtaki, Milkman, and Williams, 1968; Nijhout, 1975). It is generally accepted that the JH titer is "high" during most of premetamorphic development, yet the effects of JH are made manifest only at discrete times during this development—when ecdysone induces a molt. Ligation studies of larvae of the tobacco hornworm *Manduca sexta* suggest that in a larval molt, the effects of JH on epidermal commitment occur simultaneously with initiation of the molting process by ecdysone (Truman, 1972; Truman and Riddiford, 1974). Observations in these studies and in others (Wigglesworth, 1934; Fukuda, 1944) suggest indirectly that in premetamorphic molts the JH titer, in fact, may be very low at the time of prothoracicotropic hormone (PTTH) release, just prior to the critical period for ecdysone secretion. Other reports suggest that substantial titers of ecdysone and of JH do not occur simultaneously even in a larval molt (Patel and Madhavan, 1969).

Much has been deduced about JH titers from indirect measurements of cytological, size, or activity changes in the corpora allata (CA) (see Doane, 1973), but there are difficulties with such methods (Williams, 1961; Johnson and Hill, 1973a) and direct determination of hemolymph titers is desirable. The tobacco hornworm is especially suited for such an investigation of hemolymph titers of JH during larval development. The "gating" of PTTH release by photoperiod (Truman, 1972) allows one to select developmentally synchronous groups of animals from which to obtain hemolymph for analysis. Titers observed can be correlated precisely with developmental events relative to the release of PTTH and ecdysone. Finally, the JH content of hemolymph samples can be analyzed in a bioassay system using assay animals of the same species and stage as those which provided the hemolymph.

This report describes the determination of JH titers in hemolymph during the fourth and fifth instars of wild-type *Manduca* larvae, by means of a sensitive and quantitative JH bioassay utilizing larvae of the *black* mutant (Safranek and Riddiford, 1975).

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MATERIALS AND METHODS

Experimental animals

Both wild-type and *black* mutant larvae of *Manduca sexta* were reared in a short day photoperiod (12L:12D) at 25° C, as described by Truman (1972). Times of day will be referred to arbitrary zeitgeber time (AZT) (Pittendrigh, 1965; Truman, 1972), with lights-off and the beginning of a new day set at 24.00 AZT. Fourth instar larvae were staged as described by Truman (1972).

The black *mutant larval pigmentation assay*

Unligated fourth instar larvae of the *black* mutant (Safranek and Riddiford, 1975) show melanization of the cuticle similar to that in wild-type fourth instar larvae neck-ligatured during a critical period following initiation of the last larval molt (Truman, 1972). Topical application of JH can prevent melanization, and larvae are maximally sensitive to JH when head capsule slippage begins (Truman, Riddiford, and Safranek, 1973; Safranek and Riddiford, 1975). Synchronous fourth instar *black* larvae within a half hour of this slippage were used for the assay; under our conditions this event occurred between four and nine hours after lights-on.

Larvae were anesthetized by submersion in water for 15–20 minutes prior to assay. For application of a test solution, a disposable microliter pipette (Micropet, Clay-Adams) was used. Anesthetized larvae were swabbed dry, and one or two μl of the test solution was applied by spotting on one side between the third and fourth abdominal spiracles. After a few seconds, the solvent had evaporated, and larvae were returned to individual plastic cups without food. Larvae were scored on the third or fourth day after the hormone application, since the full extent of melanization was not apparent until a day or so after ecdysis to the fifth instar.

Selection of larvae for hemolymph samples

Larvae were selected carefully so as to be similar to one another with respect to age, time of day, and developmental stage or weight. Only Gate II larvae (Truman, 1972) were used for all extractions during the fourth instar (except for larvae on day 0, immediately following ecdysis from the third instar). Table I summarizes the selective criteria used for larvae at each time-point during the fourth and fifth larval instars. For extractions from ligated larvae, day 2 fourth instar larvae (Gate II) were neck- or abdomen-ligatured at 23.30 AZT prior to the initiation of molting, and the portion anterior to the ligature cut away. Ligated larvae were kept at 25° C until blood collection.

Collection of hemolymph

Larvae were anesthetized with carbon dioxide for 5–15 minutes. Until the time of head capsule slippage, the tip of the abdominal horn was cut off to bleed larvae, whereas with older larvae the tips of the second or third pair of abdominal

TABLE I

Criteria used to select Manduca sexta *larvae for hemolymph collection.*

Stage	Description of Stage	Time (AZT) of Weighing	Weight Range (gm)	Time (AZT) of Blood Collection
IV, d0	Newly ecdysed (day 0) fourth instar larvae	—	—	17.00–22.00
IV, d1	Day 1 fourth instar larvae, about 27–30 hrs before time of PTTH release	19.30–21.30	0.40–0.60*	19.30–21.30
IV, d2, −10h	Day 2 fourth instar larvae, about 10 hrs before the time of PTTH release	13.30–14.30	0.60–0.85	13.30–14.30
IV, d2, −½h	Day 2 fourth instar larvae, about 0.5 hr before the time of PTTH release	22.30–23.30	0.70–1.10	23.15–23.45
IV, d3, +3h	Day 3 fourth instar larvae, about 2.5-3 hr after the time of PTTH release	22.00–23.00	0.75–1.10	02.45–03.15
IV, d3, +12h	Day 3 fourth instar larvae, about 12 hrs after initiation of molting; spiracle apolysis just evident	—	—	12.15–13.00
IV, d3, +18h	Day 3 fourth instar larvae; spiracle apolysis advancing; headcap well-tucked or just beginning to slip	—	—	18.00–19.00
IV, d3, +23h	Day 3 fourth instar larvae; headcap slipped at least past the most anterior ocellus	—	—	23.00–23.30
IV, d4	Day 4 fourth instar larvae, new fifth instar mandibles tanned pale orange to dark brown; no air in old headcap	—	—	18.30–19.30
V, d0	Newly ecdysed (day 0) fifth instar larvae	24.00	1.0–1.6	24.00
V, d1	Day 1 fifth instar larvae, about 12–18 hrs after ecdysis	17.00–20.00	1.8–2.4	17.00–20.00
V, d2	Day 2 fifth instar larvae, about 36–48 hrs after ecdysis	19.00–20.00	2.5–3.5	19.00–20.00
V, d5	Mature fifth instar larvae, about day 4 or 5; no evidence of gut purging or exposure of dorsal vessel	15.00	9.0–10.9	15.00

* During day 1, Gate I and Gate II larvae can be distinguished with 90–95% success on the basis of their weights (Dr. H. F. Nijhout, University of Washington, personal communication).

prolegs were severed. By gentle pressure, hemolymph free of gut contents was expressed from the cut opening into an ice-cold calibrated test tube. Blood either was extracted immediately after collection, or was rapidly frozen in dry ice. Frozen blood was stored for up to two weeks at − 15° C and then thawed in ice water immediately before beginning the extraction. No differences between frozen and unfrozen samples were observed.

Glassware and reagents

All reusable glassware used in the extractions as well as Pasteur pipettes used for transferring solutions and for preparing micro-columns were doubly washed, rinsed with distilled water, doubly rinsed with acetone (Nanograde, Mallinckrodt), and oven dried. Precaution was taken to insure that disposable glassware including microliter pipettes (Micropet, Clay Adams) and micro-test tubes (6 × 50 mm culture tubes, Kimble) were kept free from contamination with juvenile hormones.

Methanol (Matheson, Coleman & Bell), anhydrous diethyl ether (MCB or Allied Chemical), and n-hexane (bp 68.7° C, range 4° C, Baker) were of A.C.S. specifications. The cyclohexane used was spectrophotometric grade (Baker). All solvents were tested periodically to insure that they remained free from JH contamination by carrying out the complete extraction procedure, or portions of it, on the appropriate volume of solvent, and assaying the resulting "extract". Activity IV alumina was prepared from neutral aluminum oxide (M. Woelm-Eschwege, distributed by Alupharm Chemicals) as described by the suppliers.

Preparation of micro-columns

Pasteur pipettes (inner diameter = 5 mm) were plugged with small pieces of glass wool (previously doubly soaked in Nanograde acetone and dried). The bores of the pipettes were filled with a 2.5 cm column of activity IV alumina followed by a 2 cm column of anhydrous sodium sulphate (Merck). Micro-columns were prepared in batches of six or seven, washed with 20–30 ml hexane and stored (filled with hexane) until use. The hexane washes were "extracted" and assayed to insure that columns were free from JH activity.

Extraction and assay procedures for hemolymph extracts

Seven ml of hemolymph were shaken with 25 ml of ether: methanol (4:1) on a vortex mixer. In a few instances, the volume of hemolymph was less than 7 ml, but never less than 6.0 ml. The mixture was centrifuged at about 150 *g* for 5 minutes (Sorvall GLC–2 centrifuge), and the upper clear phase was removed to a round-bottomed flask. The remaining phase and precipitate were re-extracted twice with ether: methanol (4:1) (total volume 50 ml), as before. The combined organic extracts were then concentrated in a rotary evaporator with bath temperature never exceeding 30° C. Eight ml of hexane were added to the residue, and, after vigorous mixing, the hexane phase was removed and passed through a micro-column (prepared as described above) and the eluant collected. The hexane extraction and column "clean-up" were repeated twice (total volume 16 ml) after which a final 5.0 ml of hexane were passed through the column. Eluants were pooled (total volume about 29 ml), concentrated, and the final residue transferred in hexane to a micro-test tube. Solvent was evaporated under a stream of nitrogen gas.

A volume of cyclohexane equal to 1/250 of the initial volume of hemolymph was added to the N_2-dried extract (*i.e.,* 24 to 28 μl). Two μl per assay animal of this solution—the "undiluted extract"—constituted the initial (1×) dilution and provided to each animal an amount of extracted JH activity equivalent to that

TABLE II

Scoring system for the black *mutant larval pigmentation assay for JH.*

Score	Description*
0	Larvae totally black, including head capsule (hc), thorax (thor) and abdomen (abd); no evidence of localized green spot at the site of hormone application.
+1	Larvae black except for a green spot at the site of hormone application; often green banding extends from the spot to the dorsal midline above the spot.
+2	Larvae black except for green spots on both sides of the segment at the site of application; spots often connected by a green band.
+3	Larvae black with green patches on segment of application and at least one adjacent segment, but may include as much as a three or four segment wide green region centering around the site of application; green region may extend dorsally on the affected segments; sometimes thor muddy colored; occasionally hc partly green.
+4	Larvae green; dorsal transverse stripes on abd moderately heavy and extend as far as the spiracles except in a one to three segment wide area centering around site of application; oblique black stripes present.
+5	Larvae nearly completely green; oblique black stripes pale, at most a few black spots; transverse stripes absent or very pale, restricted to the dorsal midline; some pink pigmentation visible in epidermis.

* See Figure 1 for a photograph of larvae representative of each response category.

from 0.5 ml of hemolymph. From the undiluted extracts, dilution series in cyclohexane were prepared. Assays were performed as described above, with applications of 2 μl cyclohexane serving as controls.

Extraction and assay of a known amount of C18JH indicated that the efficiency of the extraction procedure was close to 100% (see Fig. 2).

Juvenile hormones

Synthetic methyl 10,11-epoxy-3,7,11-trimethyl-*trans*-2,*trans*-6-dodecadienoate (C16JH) was generously provided by Dr. A. M. Ajami or purchased from Eco-Control. Synthetic methyl *cis*-10,11-epoxy-7-ethyl-3,11-dimethyl-*trans*-2-*trans*-6-tridecadienoate (C18JH) (*ca.* 90% purity) was provided by Dr. Kondo of Sagami Chemical Research Center. Stocks of hormones were stored at −15° C.

RESULTS

Scoring system for the black *mutant larval pigmentation assay*

From the responses of over 250 *black* larvae to various doses of C16JH, the scoring system in Table II was devised. Examples of the responses described in Table II are shown in Figure 1.

Two sorts of "false positives" occurred with low frequency and were eliminated from all tabulations. The first type comprised larvae with a bluish (rather than

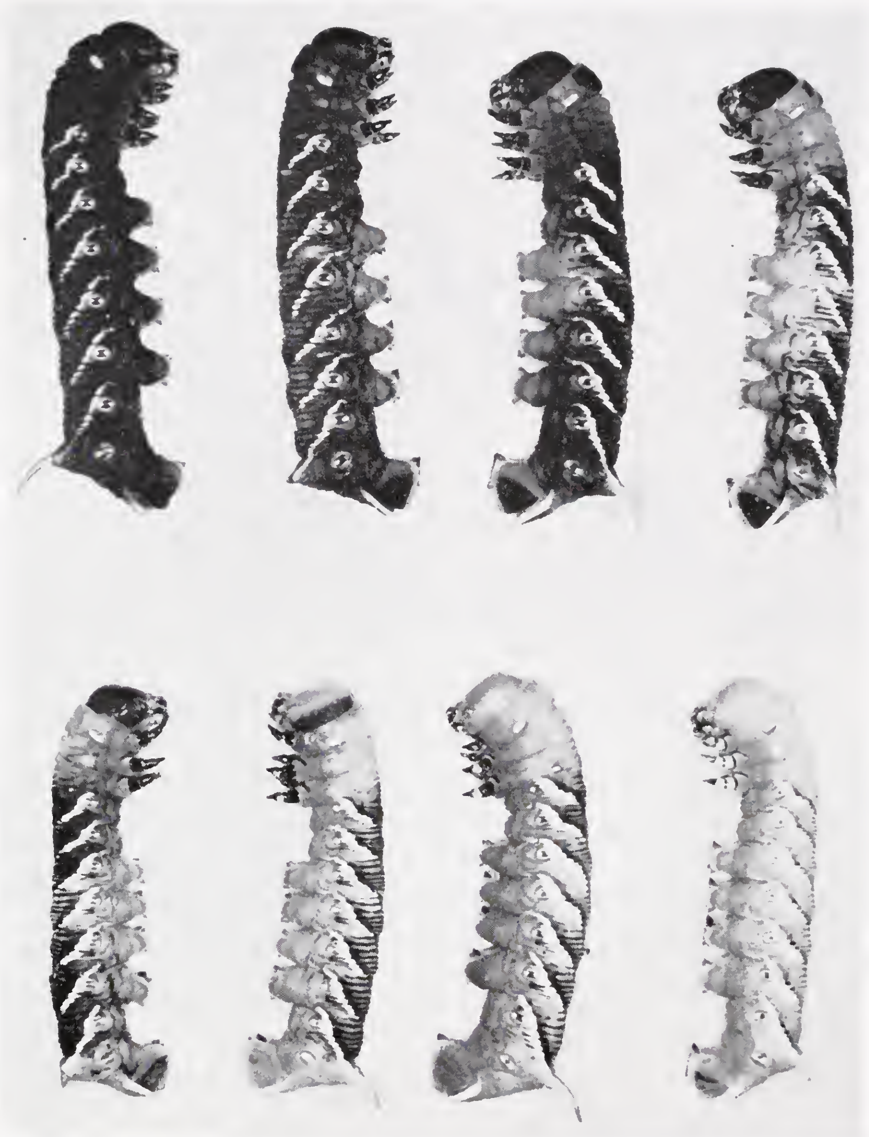

FIGURE 1. Fifth instar *black* larvae following application of various amounts of juvenile hormone at the time of head capsule slippage in the fourth instar. Assay scores are, left to right: upper row, 0, +1, +1 or +2, +3; lower row, +3, +4, +4, +5.

green) "wound" spot at or near the site of hormone application. In response to irritation by cyclohexane, the cuticle had apparently been damaged. If such larvae were gently stretched, the "wound" cuticle would tear. Tearing did not occur with comparable stretching of a "+1" larva. The second type of false-positive

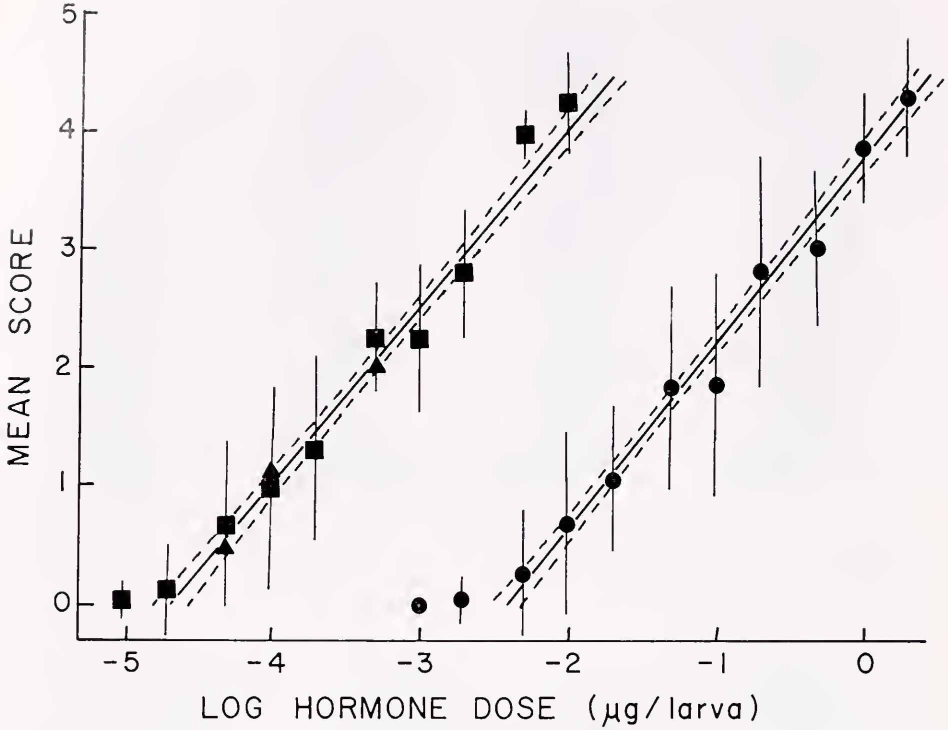

Figure 2. Dose-response relations for C16- and C18JH in the *black* mutant pigmentation assay. Squares represent C18JH; circles, C16JH; solid lines, best-fit lines determined by linear regression of individual scores onto dose, at all doses for which mean score > 0.1 (slopes 1.50 and 1.54, respectively); dotted lines, 95% confidence limits. Correlation coefficients are C18JH, $R = 0.91$; C16JH, $R = 0.88$. X-intercepts are C18JH, 2.1×10^{-5} μg; C16JH, 3.8×10^{-3} μg. Each point represents the mean score of 25–37 larvae; the bars indicate ± one standard deviation. Triangles show mean scores for 3 dilutions of C18JH "extracted" and assayed according to the procedures in Methods (9 ± 1 assay larvae per point).

was similar to that observed in the original pigmentation assay utilizing neck-ligated wild-type larvae (Truman *et al.*, 1973) in that the larval cuticle appeared hormone application.

Dose-response relations for juvenile hormones

Figure 2 shows the mean scores obtained for different doses of C16- and C18JH. The lines drawn through each set of points are the least squares lines calculated by regression of individual scores onto the $\log_{10}$ hormone dose. The correlation coefficients ($R = 0.91$ and $R = 0.88$ for C18- and C16JH, respectively) indicate that these lines well represent the experimental data. As expected from results in other *Manduca* JH assays (Nijhout and Riddiford, 1974; Riddiford and Ajami, 1973), C16JH is considerably less active than C18JH. In spite of a nearly 200-fold difference in the activities of these two hormones, the slopes of their respective dose-response curves are indistinguishable, within experimental error. The third known naturally-occurring juvenile hormone (C17JH) was unavailable at the time of this investigation, but C17JH has been shown to have an identical activity to C18JH in three other *Manduca* JH assays (pupal injection assay, Riddiford and Ajami, 1973; pigmentation assay, Truman *et al.*, 1973; egg maturation assay, Nijhout and Riddiford, 1974).

Concentration of juvenile hormone in the hemolymph

Figure 3 shows the relative JH concentration in the hemolymph from ecdysis to the fourth instar through the first third of the fifth instar. Mean scores were converted to JH concentrations using the dose-response relations shown in Figure 2. Since extremes obtained by extrapolation within the 95% confidence intervals for the least squares lines differ by less than 0.15 log unit (Fig. 2), this aspect of error has been ignored in the calculation of JH equivalents. The JH titer declines slowly until after the release of PTTH and ecdysone initiating the molt to the fifth instar; then the concentration decreases sharply. T-tests indicated that the observed differences among the concentrations determined at various times on day 2 of the fourth instar are not significant ($0.6 > P > 0.3$). Immediately after ecdysis to the fifth instar the JH titer has increased sharply, then by day 1 has declined and is maintained at a constant level through day 2. By the end of the feeding stage (V, d5) the concentration has declined to less than 1/16 of the V, d2 value.

Adult female *Manduca* CA can secrete *in vitro* C16-, C17- and C18JH (Judy, Schooley, Dunham, Hall, Bergot, and Siddall, 1973; Reibstein and Law, 1973), but to date only C16JH has been reported to have been detected in fourth instar larval blood of *Manduca* (Judy *et al.*, 1973; Dr. K. J. Judy, Zoecon Corp., personal communication). Because a very small amount of C17- or C18JH, in addition to any C16JH, could account for a substantial proportion of the extracted JH activity, it is best not to assume that the extracted activity is equivalent only to C16JH. In Figure 3, the right-hand ordinate expresses the JH concentrations in ng/ml C18JH equivalents. JH concentrations in terms of C16JH equivalents would be nearly 200-fold greater. The slopes of the standard curves for C16- and C18JH are identical, and thus the *relative* JH titers are the same whether based on C16- or C18JH.

Rate of degradation of endogenous juvenile hormone

The level of JH observed in hemolymph at intervals following ligation can provide an estimate of the actual rate of "degradation" (including excretion or inactivation) of JH in the hemolymph, since ligation removes the sources of JH (the CA). At varying times after ligation, blood was collected, extracted and assayed just as for intact larvae. The JH titer in intact larvae of the same age (stage IV, d2,-$\frac{1}{2}$h in Table I) was considered to be the "0 hour" level of JH. Figure 4 shows the relative concentration of JH in the hemolymph after ligation. The line drawn through the points indicates a half-life ($T_{\frac{1}{2}}$) for endogenous JH in the hemolymph of about 1.5 hours.

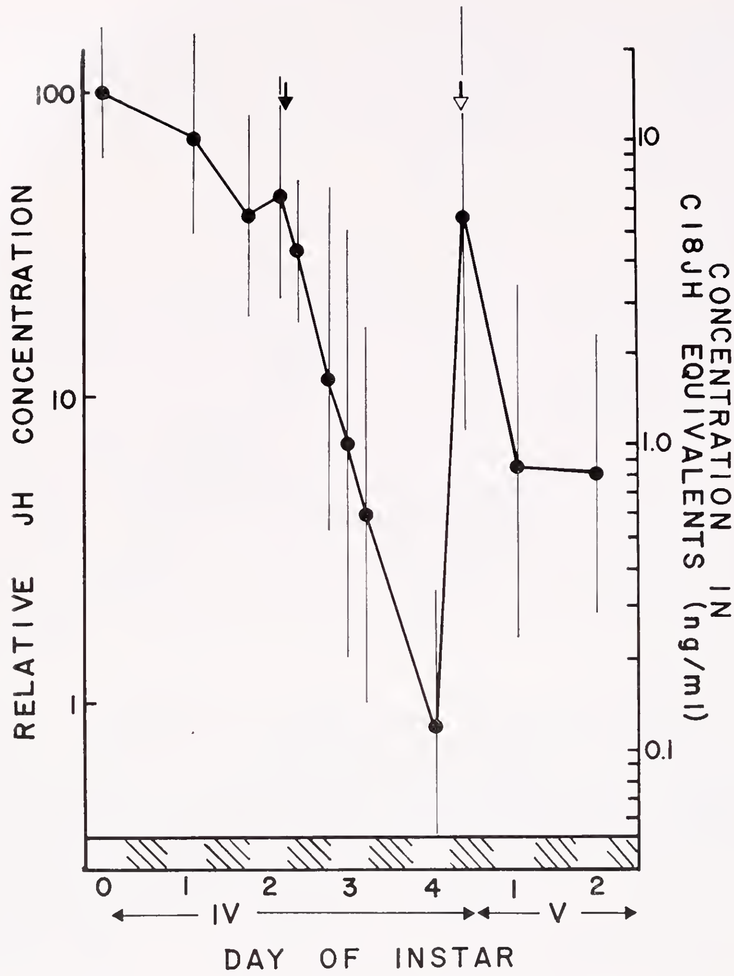

FIGURE 3. Juvenile hormone titer in *Manduca* larvae after ecdysis to the fourth instar through the first third of the fifth instar. The JH concentration at each stage was calculated from the mean scores of initial dilutions of extracts. Each point is based on 3 to 5 extractions and about 25 assay larvae; bars indicate ± one standard deviation; filled arrow, mean time of PTTH release for Gate II larvae as determined by Truman (1972); open arrow, mean time of ecdysis of Gate II larvae to the fifth instar; cross-hatch represents darkness.

Knowing the half-life, one can estimate the rate constant "a" in the expression for the rate of decay, $V_D = dx/dt = -ax$, where "x" is the amount of JH and "t" the time. Integration of the rate expression, from $x = x_0$ to x, and $t = 0$ to t, yields the expression, $x = x_0e^{-at}$, where "x_0" is the initial amount of JH at $t = 0$. Since $x = x_0/2$ at $t = T_{\frac{1}{2}} = 1.5$ hours, then $a = 0.46$ hours^{-1}. Thus the rate expression is $V_D = -x\ (0.46)$ hours^{-1}. Assuming that this rate constant is characteristic of intact fourth instar larvae during the latter part of day 2, one can

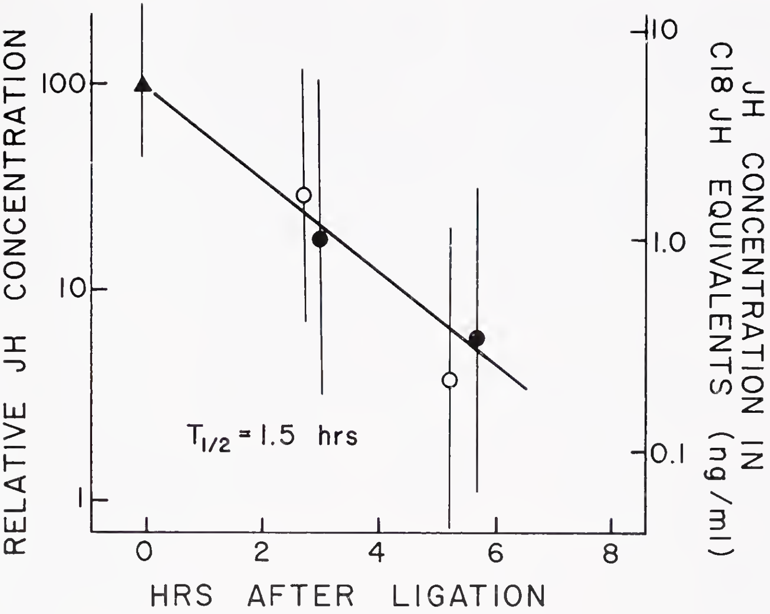

FIGURE 4. Decay of juvenile hormone activity in hemolymph following neck- or abdomen-ligation of fourth instar larvae. JH concentrations were calculated from mean scores of initial dilutions of extracts. Triangles represent unligated day 2 fourth instar larvae (stage IV, d2, −1/2h); filled circles, day 2 fourth instar larvae abdomen-ligated at 23.30 AZT; open circles, day 2 fourth instar larvae neck-ligatured at 23.30 AZT. Points for ligated larvae are based on two extractions and 11–16 assay larvae; bars indicate ± one standard deviation. By 20 hours after ligation, no detectable JH activity remained in either neck- or abdomen-ligated larvae.

estimate the rate of production (synthesis and secretion) of JH which would be required to maintain the titer at the fairly constant level observed during day 2 (Fig. 3). At such a steady state, the rate of production, V_S, must be equal and opposite to the rate of decay, V_D. Thus during this period, $V_S = -V_D = x\ (0.46)$ hours^{-1}, where "x" is the steady state level of JH. The concentration of JH during this period in intact larvae was approximately 6 ng/ml C18JH equivalents. Total blood volume of larvae which weighed 0.7–1.0 gm was estimated to be roughly 0.4 ml. From these data the steady state level of JH was determined to be 2.4 ng C18JH equivalent per larva, corresponding to $V_S = 1.1$ ng/hour per pair CA (C18JH equivalents). In terms of C16JH equivalents, $V_S = 2 \times 10^2$ ng/hour per pair CA.

DISCUSSION

The use of *black* larvae (instead of neck-ligated wild-type) and the revised scoring system (Table II) facilitated the removal of false positives and resulted in log-linear dose *vs.* response curves over most of the range of the assay, particularly in the lower range, with standard hormones. The departure from log-linearity at high doses (mean score > 4.5) was not of concern for purposes of assay of hemolymph extracts.

For comparison of the activities of different test materials, the dose-response relations may be characterized by the dose (or dilution) required to give a criterion score (*e.g.,* a mean score of 2.5). Since assay responses are assigned numerical value on the basis of an arbitrary graded scale from 0 to 5 (Table II), a mean score of 2.5 would be analogous to 50% maximal response (or "I.D. 50") (Staal, 1972). This approach is useful for standard hormones, but could only be applied to hemolymph extracts for which the initial assayable dilution gave a mean score ≥ 2.5. Less active extracts could not be compared unless larger initial volumes of blood were extracted. Instead, extracts were compared directly on the basis of the mean scores obtained with a given dilution. By choosing the initial (1×) dilution for this purpose, the applicability of the assay even to the relatively inactive extracts was maximized. In comparing mean scores of initial dilutions of extracts with a dose-response relation established for standards, one must assume that the extracted (unknown) substance(s) and the chosen standard behave similarly in the assay. Since the dose-response relations for both C16- and C18JH were parallel and since larval hemolymph may contain C16- and C17JH, this was a reasonable assumption. As further justification for this assumption, extracts were analyzed by two different approaches making use of assays of dilution series (Fain, 1975). Extract activities were compared on the basis of the dilution required to yield an essentially "0" score (mean score < 0.15). Also, only "+1" responses were considered and extracts were compared on the basis of the dilution corresponding to the 50% point in the distribution of this response. The relative JH titers calculated by these methods were substantially in accord with those calculated directly from mean scores of initial dilutions (Fig. 3). Thus, under the conditions employed, mean scores of initial dilutions of extracts of larval blood provide reasonable estimates of relative JH titers.

In Figure 4, the half-life ($T_{\frac{1}{2}}$) of endogenous JH in the hemolymph after ligation was estimated to be about 1.5 hours. This value agrees with that determined by Johnson and Hill (1973b) for the decay of endogenous JH in the hemolymph following surgical removal of the CA in adult male *Locusta,* and with that of Reddy and Krishnakumaran (1972) for the loss of JH activity in early last instar *Galleria* larvae following injection of C18JH. In *Manduca,* an even shorter half-life (about 0.5 hour) for both endogenous (Nijhout, 1975a) and exogenous (Slade and Zibitt, 1972) JH has been observed in fifth instar larvae, and for exogenous JH in pupae (Ajami and Riddiford, 1973). This rapid decay of endogenous JH from the hemolymph contrasts with the apparent persistence of the effects of JH in isolated abdomens of young fifth instar (Nijhout, 1975a) and of fourth instar *Manduca* larvae and in fragments of fourth instar epidermis cultured *in vitro* (Fain and Riddiford, 1973; Fain, 1975). Fourth instar epidermis of *Manduca* remains capable of a larval response to exogenous β-ecdysone, both *in vivo* and *in vitro,* for many hours after removal of CA.

When CA from adult female *Manduca* were cultured *in vitro* with radio-labelled methionine, both C16- and C17JH—labelled in the ester methyl—were recovered from the medium (Judy *et al.,* 1973). The data from this study provide information on average rates of synthesis and secretion over a 24 hour period. These rates are about 0.045 ng C16JH/hour and 0.065 ng C17JH/hour per pair CA or a total of about 0.11 ng "JH"/hour per pair CA. Reibstein and Law (1973), using homogenized *Manduca* adult female CA incubated for 12 hours with labelled S-adenosylmethionine, reported at best a linear rate of synthesis of JH (labelled material in the "JH zone" of their TLC) of about 0.04 ng/hour per pair CA. But in cultures of *Manduca* CA supplemented with possible precursors for the sesquiterpenoid skeleton, higher rates of JH production have been observed, as well as changes in the ratio of the various JH's produced (Dr. H. K. Dahm, Texas A & M University, personal communication). Thus, the biosynthetic capacity of CA under some *in vitro* conditions may be restricted by sub-optimal substrate availabliity (Pratt and Tobe, 1974), and the *in vitro* synthetic rates and products may not be indicative of those *in situ* (see Ajami, 1974).

The rate of synthesis required to maintain the observed JH titer during day 2 of the fourth instar in *Manduca,* in terms of C18JH equivalents (1.1 ng/hour per pair CA) is only about 10-fold greater than the average rate of synthesis of total JH reported by Judy *et al.* (1973). But the required rate of synthesis in terms of C16JH equivalents (200 ng/hour per pair CA) is very much greater than the *in vitro* rates observed. Quantitative unambiguous determination of the amounts of different JH's in the hemolymph and of the *in vivo* secretory products of the CA during development will be required to resolve definitively the questions raised by these differences. Yet the results presented here suggest that C17- or C18JH may account for most of the observed JH activity in the hemolymph of fourth instar larvae.

Titers of JH in principle may be regulated in a variety of ways (see de Wilde *et al.,* 1971). Recent findings have stressed the importance of changing rates of degradation (or inactivation) of JH in altering hormone levels in *Manduca* (Weirich, Wren, and Siddall, 1973; Sanburg, Kramer, Kézdy, Law, and Oberlander, 1975; Sanburg, Kramer Kézdy, and Law, 1975), especially since a JH-binding protein in the hemolymph of *Manduca* larvae (Kramer, Sanburg, Kézdy, and Law, 1974) affords bound JH nearly complete protection *in vitro* against hydrolysis by general esterases, but not by JH-specific esterases (Sanburg, Kramer, Kézdy, and Law, 1975; Sanburg, Kramer, Kézdy, Law, and Oberlander, 1975). Although JH-specific esterases may be important in elimination of JH from the hemolymph at the end of the feeding phase of the fifth instar, the relation of such esterases to JH titer changes during the fourth instar is uncertain. If the JH activity in young fourth instar larvae were due primarily to C17- or C18JH, the JH concentration at this time (*e.g.,* day 2) would be about 2×10^{-8} M. Nearly all of this JH should be associated with JH binding protein, if the hemolymph concentration and dissociation constant of binding protein in fourth instar larvae are the same as reported for fifth instar larvae (concentration: 8×10^{-6} M; $K_d = 3 \times 10^{-7}$ M) (Kramer *et al.,* 1974). Almost no detectable JH-specific esterase activity has been observed in fourth instar hemolymph, although general esterase activity is present and appears to increase slightly on day 3 of the instar (Sanburg, Kramer, Kézdy, and Law, 1975). Since the rate of inactivation of JH on day 2 is already quite rapid ($T_{\frac{1}{2}} = 1.5$ hrs), the decline in the JH titer that follows the initiation of the last larval molt may reflect changes in the rate of production of JH by the

CA, rather than alterations of inactivation rates. The observations of Williams (1961) on CA activity of fourth instar *Cecropia* larvae support this suggestion.

In terms of the relative JH concentration during the last two larval instars, one can conclude the following. The JH concentration is highest immediately after ecdysis to the fourth instar, and the level remains high until after the endocrine initiation of the molt to the fifth instar. During this period there may be small (and perhaps physiologically significant) fluctuations in the JH titer. Following the initiation of molting, the JH concentration drops considerably (about 30-fold) over the last two days of the fourth instar. Immediately after ecdysis to the fifth instar, the JH concentration has risen significantly to nearly the level on day 2 of the fourth instar. But by the next day (day 1), it has decreased and remains at a plateau level through the following day (day 2). Nijhout (1975a) has confirmed a comparable low titer on day 2 of the fifth instar. By the end of the feeding phase of the fifth instar, the concentration has dropped to essentially undetectable levels as was observed also by Nijhout and Williams (1974).

Truman (1972) obtained primarily precocious pupations after neck ligature of day 2 fourth instar *Manduca* larvae at the time of PTTH release; but when neck-ligature occurred 1.5 hours later, he observed larval molting. Therefore, he concluded that following PTTH release a surge of JH was necessary for the larval molt. Yet the data presented here indicate that a relatively high and constant titer of JH persists throughout the day preceding PTTH release. Nijhout (1975b) has shown by ecdysone perfusions that the level of JH in young fifth instar *Manduca* larvae is sufficient to allow complete or nearly complete larval molting in response to exogenous ecdysone. In the present study, the JH titer in young fifth instar larvae of comparable age (stage, V, d2) was 6–8-fold lower than that observed late in day 2 of the fourth instar. A transient decline in the JH titer of 8-fold or more at the time of PTTH release in the fourth instar is unlikely to have been masked by averaging of differences among individual larvae. The photoperiod synchronization of initiation of molting (Truman, 1972) and selection of developmentally comparable larvae for extractions argue against such artifacts of averaging. In addition, nearly all of larvae neck-ligatured at 23.30 AZT eventually pupate (Fain, 1975), so extracts were made at a time appropriate to have detected a drop in JH if one occurred. It is evident also that substantial titers of both JH and ecdysone occur together at the time that the last larval molt is initiated. The significance of the formation of precocious pupae after neck ligation at the time of PTTH release will be discussed in another paper (Fain and Riddiford, in preparation).

Williams (1961) showed that during larval development in *Hyalophora cecropia* the CA undergo cyclical changes in activity. Similarly, Krishnakumaran and Schneiderman (unpublished, cited in Patel and Madhavan, 1969) found that the extractable JH activity (in *Galleria* Units) in whole larvae of *Samia cynthia ricini* was high immediately following ecdysis to the fourth instar and decreased during the course of the instar up to the time of ecdysis. As with Cecropia CA activity (Williams, 1961), the JH titer in young fifth instar Cynthia larvae was considerably higher than that at the end of the fourth instar. The present observations on *Manduca* are in accord with these findings.

By contrast, Bartelink (unpublished, cited in de Wilde *et al.,* 1971) found that the JH concentration (in *Galleria* Units) in hemolymph of *Philosamia cynthia* larvae was essentially constant throughout the fourth instar and dropped about 10-fold at the beginning of the fifth instar. This discrepancy between Bartelink's findings and our observations on *Manduca* may reflect differences in experimental animals, in the assays employed (*Galleria* wax wound *vs. Manduca* pigmentation), or in the procedures used to select larvae for blood collection. With *Manduca* it is possible to select developmentally synchronous groups of larvae before, during and after initiation of molting (Truman, 1972). Thus, in the present study, averaging of developmental differences in JH titer was minimized.

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SUMMARY

1. A sensitive and quantitative bioassay for JH is described based on the JH inhibition of cuticle melanization in larvae of the *black* mutant of *Manduca sexta.* The assay is sensitive down to about 2×10^{-5} μg (20 pg) C18JH.

2. Using this assay, the JH titers in hemolymph of larvae of *M. sexta* from ecdysis to the fourth instar through the first third of the fifth instar have been estimated. The JH titer is highest immediately after ecdysis to the fourth instar and remains relatively high until after initiation of the last larval molt. During the time that molting is initiated, the JH concentration is about 2×10^{-8} M in terms of C18JH equivalents. Following initiation of molting the titer drops about 30-fold.

3. Immediately after ecdysis to the fifth instar, the JH titer is again high (nearly the same as seen at the initiation of the molt), then by day 1 has declined sixfold and remains constant through day 2. In mature fifth instar larvae, the titer has dropped again to essentially undetectable levels.

4. The rate of degradation of endogenous JH in the hemolymph was estimated following neck- or abdomen-ligation of fourth instar larvae. After either type of ligation, JH activity was lost from the hemolymph with a half-life of about 1.5 hours.

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COMPARATIVE CHEMOSENSITIVITY TO AMINO ACIDS AND THEIR ROLE IN THE FEEDING ACTIVITY OF BATHYPELAGIC AND LITTORAL CRUSTACEANS [1]

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At the greater ocean depths, animals seek their prey in the virtual absence of light. These predators must therefore use other sensory stimuli to locate prey. It is likely their faculties of chemoreception and mechanoreception are of particular importance. This study attempts to clarify the role of chemoreception in the feeding activity of deeper-living crustaceans by comparing the chemosensitivity of a bathypelagic species with that of a shallower-living benthopelagic species and three intertidal species. The adopted approach examines the behavioral and electrophysiological responses of these species to a range of amino acid concentrations in an attempt to determine the thresholds of these responses.

The bathypelagic species chosen is the lophogastrid mysid *Gnathophausia ingens,* a predator of small fish and crustaceans which inhabits depths of 600–750 meters by day, spreading at night to depths of about 400–900 meters (Childress, 1969). At lesser depths, the galatheid crab *Pleuroncodes planipes* functions both as an epipelagic filter feeder and a benthic species of the continental shelf, at maximum depths of around 300 meters (Boyd, 1963). Intertidal species are represented by *Pagurus hirsutiusculus,* an anomuran crab, *Spirontocaris taylori,* a carid shrimp, and *Cancer antennarius,* a brachyuran crab.

Amino acid sensitivity in littoral crustaceans and the stimulation of feeding activity by amino acids has been demonstrated in previous investigations. Receptor thresholds of 5×10^{-5} M glutamic acid in *Carcinus maenas* dactyls (Case and Gwilliam, 1961) and 5×10^{-7} M taurine in *Homarus americanus* antennules (Ache, 1972) have been reported. Levandowsky and Hodgson (1965) reported *Panulirus argus* to initiate feeding in the presence of 5×10^{-4} M glutamic acid. This study examines chemoreceptive acuity in additional littoral species to provide a broader context in which to view the chemoreceptive abilities of deeper-living species.

An earlier investigation of receptor specificity in the *Panulirus interruptus* antennule (Fuzessery, unpublished) is included in this study as a partial basis for the choice of constituents of the amino acid mixture used as a feeding stimulant. Two general classes of receptors were found which responded maximally to either mono- or dicarboxylic amino acids, implying that a combination of these compounds would have an increased effect on feeding behavior through maximal activation of receptors with different specificities. Ache (1972) and Shepheard (1974) also found receptor specificity to different amino acids in the antennule receptors of *Homarus americanus.* McLeese (1970), and Shelton and Mackie (1971) have provided behavioral evidence that maximal feeding stimulation in crustaceans requires combinations of compounds, although these studies included other organic compounds in addition to amino acids.

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Finally, we will attempt to place the results of this study into the context of the bathypelagic environment of *G. ingens,* and examine the possible means of employment of the chemoreceptive faculties of this species in its feeding activity.

MATERIALS AND METHODS

Gnathophausia ingens was collected with a midwater trawl from basins off the coast of southern California. *Pleuroncodes planipes* was collected with a meter net at shallower depths off Baja California. The three littoral species tested were collected in tidepools on the Santa Barbara coast.

G. ingens is one of the few bathypelagic species suitable for this study, as it has been maintained in the laboratory for up to 2.5 years (Childress, 1971).

Behavioral studies

The standard feeding stimulant used in this study was an equimolar solution of L-glutamic acid, taurine and DL alpha amino-n-butyric acid. This mixture was chosen for the following reasons: (1) studies by other investigators (Hodgson, 1958; Case and Gwilliam, 1961; Case, 1964; Laverack, 1964; Levandowsky and Hodgson, 1965; and Ache, 1972) have shown these compounds are effective feeding stimulants in crustaceans, (2) a mixture rather than a single amino acid was considered to more closely approximate a situation naturally encountered by crustaceans, and (3) an earlier study of amino acid specificity in *P. interruptus* antennules indicated the presence of two classes of receptors responding maximally to either mono- (*e.g.,* aminobutyric acid) or dicarboxylic (*e.g.,* glutaminc acid) amino acids (see results). The remaining mixture component, taurine, activated both receptor types and was found to be particularly stimulatory in this and other studies (Case, 1964; Ache, 1972; Shepheard, 1974). Natural feeding stimulants (food extracts) were not used because of obvious difficulties in identifying constituents and quantifying their concentrations.

The feeding responses of the five species tested were initially observed in the presence of 10^{-3} M amino acid mixture for the purpose of describing response characteristics. These responses were categorized in a hierarchy of response intensity, and a minimal criterion for a feeding response exhibition was defined for each species. Behavioral sensitivity to the mixture was determined by observing the percentage of individuals exhibiting a feeding response in each of a range of mixture concentrations.

For the tests, individuals were placed in separate chambers containing filtered seawater. One hundred and fifty specimens of *G. ingens* and 175 specimens of *P. hirsutiusculus* were tested in 250 ml glass bowls; tests on *G. ingens* were conducted in a temperature-controlled room at 7° C, the normal ambient temperature of this species. Sixteen specimens of *S. taylori* and 90 specimens of *P. planipes* were tested in 1000 ml glass bowls. *Spirontocaris taylori* required a small clump of red algae in which to "conceal" itself; without this, this species would circle the bowl continuously. Fifteen specimens of *C. antennarius* were tested in 3 gal. aquaria; all individuals of this species had a carapace width of less than 4 inches. At least 30 min were allowed for the individuals to achieve a quiescent state (*i.e.,* no avoidance responses and a minimum of exploratory behavior) before testing. Solutions were introduced *via* syringe through air tubing at a rate of 0.5–1.0 ml/sec. An inital seawater introduction, serving both to determine the individual's response to mechanostimulation and as an attempt to adapt-out this response, was followed by either a second seawater introduction serving as control or a given concentration of the mixture. The individual was considered sensitive to the test solution if the designated feeding response was observed between the time of introduction and one minute following. The effect of chemical stimulation on a given species was considered to be the percentage of individuals responding to a given concentration minus the percentage responding to the seawater control.

In the same manner, the responses of *G. ingens* were also observed in the presence of additional individual amino acids, representing structural groups not included in the mixture, L-phenylalanine, L-arginine, and hydroxyproline, and to trimethylamine hydrochloride and betaine hydrochloride. The responses of *P. hirsutiusculus* to the individual components of the mixture were observed to determine the relative stimulatory effects of single and combined amino acids. Finally, the effect of simultaneous chemo-tactile stimulation was tested in *G. ingens* by touching blotter paper permeated with a given mixture concentration to the thoracic legs for one second.

With the exception of *G. ingens*, individuals were not without food more than five days before testing. All tests on *G. ingens* were done within 10 days of capture, during which time they were not fed.

Electrophysiological studies

The response thresholds of the receptors of *G. ingens* dactyls and antenna, and *C. antennarius* dactyls and third maxillipeds were examined electrophysiologically for comparison with the behaviorally determined thresholds of these species. *Panulirus interruptus* antennular receptors in both the medial and lateral flagella were examined for specificities to different amino acids, but thresholds were not investigated. Sensory activity was recorded from subdivided axon bundles dissected from ablated appendages, using chlorided silver wire electrodes in conjunction with standard recording instrumentation. Responses from *P. interruptus* antennules were recorded on kymograph film; all other receptor activity was integrated *via* a strip-chart recorder. Appendages were situated in a trough isolated from the exposed fibers by a rubber dam. Exposed fibers were submerged in filtered sea water; appendges were kept moist by periodic washes. Test solutions were applied to the appendages *via* syringe through a 20 gauge hypodermic needle.

In the *P. interruptus* specificity investigation, the introduction of the various amino acids (0.01 M) was interspersed by seawater washes to reduce receptor adaptation. The first amino acid tested was reintroduced at the end of the test series to compare response similarities. A loss of sensitivity to the concluding amino acid invalidated the series. Response intensities were calculated by counting the filmed impulses.

TABLE I

Percent of individuals responding to amino acid mixture.

	Control	Concentration (M)											
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}
Pagurus hirsutiusculus	(13) 8	(48) 98	(20) 80	(67) 55	(24) 42	(15) 33	(32) 16	—	—	—	—	—	—
Spirontocaris taylori	(18) 16	—	—	(18) 95	(30) 80	(12) 67	(12) 58	(12) 42	—	—	—	—	—
Gnathophausia ingens	(53) 9	(49) 72	(48) 43	(40) 44	(18) 44	(16) 31	(18) 44	(18) 33	(34) 38	—	(18) 11	—	—
G. ingens chemo-tactile response	(54) 48	(18) 100	(18) 72	—	—	(18) 67	(18) 61	—	—	—	—	—	—
Cancer antennarius	(31) 13	—	(16) 100	(27) 89	(19) 84	(27) 64	(15) 73	(8) 63	(8) 88	(8) 38	(8) 50	—	—
Pleuroncodes planipes	(32) 13	—	—	(15) 100	(31) 97	(16) 69	(31) 61	—	(23) 43	—	(16) 44	—	(22) 18

(X) = Number of tests. (—) = No tests.

In the threshold studies, a series of amino acid mixture concentrations was introduced, beginning with the lowest concentration. As fiber bundles were rarely subdivided to single receptor units, all chemoreceptive activity was judged against mechanoreceptive activity evoked by sea water. Response intensities were calculated by manually planing the area beneath the integrated response curves during the first 10 seconds. The mechanoreceptive activity of a given preparation was subtracted from the chemoreceptive activity.

RESULTS

Chemoreceptive acuity based on behavioral criteria

For each of the five species tested, feeding responses elicited by the mixture are listed below in the approximate sequence in which they occur, beginning with responses of least intensity and progressing to those which more clearly indicate awareness of a chemical presence representing potential food. Variations of these sequences occurred, as when in the presence of a high concentration, a response of greater intensity was immediately elicited, rather than a progression. For each species the response or combinations of responses judged to be a minimum criterion of a feeding response is stated. These responses represent apparent attempts to secure or ingest food simulated by amino acids. The percentage of individuals exhibiting a feeding response at each range of mixture concentrations is presented in Table I and Figure 3. Also tabulated are the frequencies of response of *G. ingens* to additional individual amino acids and related compounds (Table II) and the responses of this species to chemo-tactile stimulation (Table I).

Feeding responses in the presence of amino acid mixture

Gnathophausia ingens. (1) Anterior endopodites are moved in a circular motion over the mouth. This motion appears to be related to filter feeding. (2)

TABLE II

Percentage of Gnathophausia ingens *individuals responding to amino acids and related compounds.*

Compound	Molarity	N	% Responding
Betaine hydrochloride	10^{-4}	17	88
	10^{-6}	17	65
L-Arginine	10^{-3}	34	65
	10^{-5}	34	44
Hydroxyproline	10^{-3}	17	59
	10^{-5}	17	35
Phenylalanine	10^{-3}	17	41
	10^{-5}	17	35
Trimethlamine oxide	10^{-3}	17	29
	10^{-5}	17	24
Control	—	53	9

Endopodites are partially extended in an unconcerted manner. (3) Endopodites are extended approximately perpendicular from the body in a concerted motion, in the manner of reaching. (4) The abdomen is arched ventrally, either perpendicular to the body or with the telson almost in contact with the mouth. This is perhaps a method of entrapping prey against the ventral body surface. A feeding response was defined as (3) a concerted endopodite extension.

Pleuroncodes planipes. (1) Antennae are oriented toward the stimulus, and the antennule flicking rate is increased. (2) The third pair of maxillipeds are rubbed together. (3) The third pair of maxillipeds are outstretched and brought back to the mouth. This is probably filter feeding activity. (4) The chelipeds are brought to the mouth. (5) The substrate is prodded with the chelipeds or periopodites. (6) A capture response is executed in which the periopodites and/or chelipeds are brought to the ventral body surface in the manner of entrapment. A feeding response was defined as (4) as the motion of bringing the chelipeds to the mouth.

Cancer antennarius. (1) Antennal orientation motion and antennule flicking rate is increased. (2) Periopodites are extended in the direction of the stimulus and/or used to prod the substrate, sometimes followed by a slow search. (3) The third maxillipeds are flared and set into a lateral motion. (4) The chelipeds are brought to the mouth. (5) A capture response is executed involving grabbing and/or pouncing upon suspected prey and entrapping against the ventral body surface. A feeding response was defined as (3) maxilliped activity in conjunction with cheliped activity and/or a capture response. Maxilliped activity alone did not constitute a feeding response.

Spirontocaris taylori. (1) Antennal motion is increased, and the antennules are erected and their flicking rate increased. (2) The third maxillipeds are rubbed together. (3) The substrate is prodded with the periopodites, with these legs moving rapidly up and down in a characteristic asynchronous fashion. (4)

There is an increase in the intensity of (3), with the shrimp leaving its original position in a slow search. A feeding response was defined as (3) the rapid up and down motion of the periopodites.

Pagurus hirsutiusculus. (1) Antennal motion and antennular flicking rate is increased. (2) The third maxillipeds are extended. (3) The substrate is prodded with the periopodites and/or chelipeds. (4) The chelipeds are brought to the mouth. (5) A capture response is executed involving rapid cheliped extension, scissoring or the cheliped pincers, and grabbing motion. A feeding response was defined as (4) the motion of bringing the chelipeds to the mouth.

The same behavioral criteria applied to the additional tests on *G. ingens* in response to additional individual amino acids and related compounds, and to chemo-tactile stimulation. They also applied to tests on *P. hirsutiusculus* responses to the individual components of the amino acid mixture.

Feeding response characteristics were the same in all species to both the amino acid mixture and actual food. That these species demonstrated feeding behavior to amino acids in the absence of visual cues indicates a high degree of chemical control over this activity. Even in orientation of actual food, visual cues appear to play a comparatively minor role. Only *S. taylori*, an active intertidal shrimp, demonstrated visual orientation by occasionally intercepting pieces of food as they sank from the surface. The most commonly observed orientation to food in the species examined was a slow search apparently governed by chemical cues and effected by leg extension and, in the benthic species, prodding of the substrate in the general direction of the food.

Electrophysiological results

Panulirus interruptus—receptor specificity. Antennular receptors in *P. interruptus* demonstrated a specificity to different amino acids. Different preparations responded maximally to either monocarboxylic (*e.g.*, glycine and DL-alpha amino-

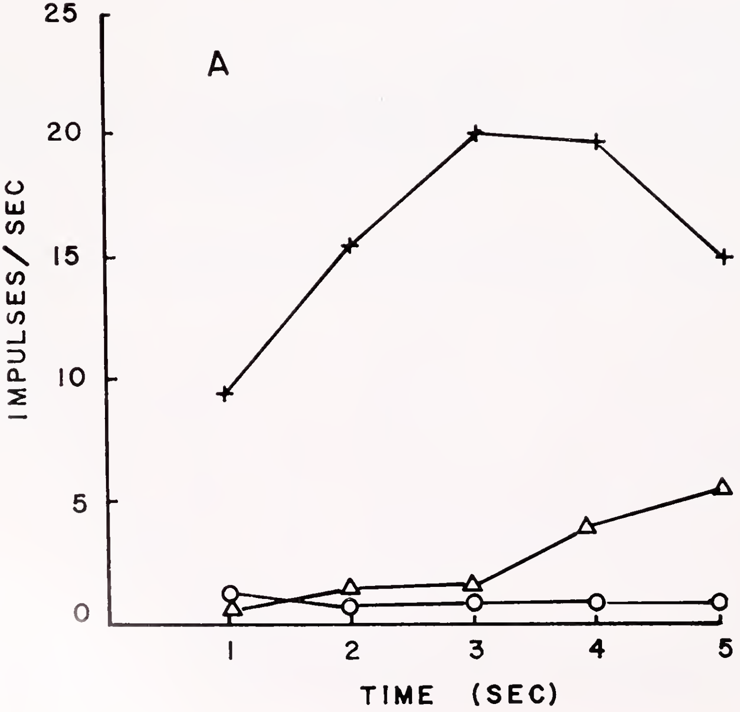
TABLE III

Percent of Pagurus hirsutiusculus *individuals responding to amino acid mixture and individual mixture components.*

	Molarity	N	% Responding
Mixture	1.5×10^{-2}	48	98
	1.5×10^{-4}	67	55
Taurine	1.5×10^{-2}	32	88
	1.5×10^{-4}	32	44
Glutamic Acid	1.5×10^{-2}	31	65
	1.5×10^{-4}	30	23
Aminobutyric Acid	1.5×10^{-2}	30	67
	1.5×10^{-4}	31	19
Control	—	13	8

n-butyric acid) or dicarboxylic (*e.g.*, L-glutamic and L-aspartic acid) amino acids, indicating the presence of at least two general classes of receptors. Responses to both groups of amino acids in a given preparation was more commonly encountered, but these responses generally appeared to be the product of a greater number of receptor units in the recorded population, implying that both receptor types may have been present, or that there are also receptors with broader specificities. Of 19 preparations selected for consistency of response to given amino acids throughout a test series, 11 responded maximally to either mono- or dicarboxylates. All 11 are medial flagellum preparations. Figure 1 represents the average number of impulses per second elicited by four preparations responding maximally to monocarboxylates (A) and seven to dicarboxylates (B). The averages are a pooling of responses to glycine and DL-alpha amino-n-butyric acid, and to L-aspartic acid and L-glutamic acid. As also indicated (Fig. 1A and B), taurine, a sulfur-substituted amino acid, activated both receptor types but appeared to be more stimulatory to receptors responding maximally to dicarboxylates. Figure 2 illustrates examples of recordings from two antennular preparations from which these data were calculated.

Gnathophausia ingens—chemoreceptive acuity. Dactyl and antennal receptors were tested for responses to mixture concentrations ranging from 1.5 × 10^{-2} M to 5 × 10^{-10} M. The response threshold of dactyl receptors was 6 × 10^{-8} M, that of the antennae 5 × 10^{-7} M. In both appendages, response intensity and duration increased with concentration. Responses to 10^{-6} M generally adapted within 15



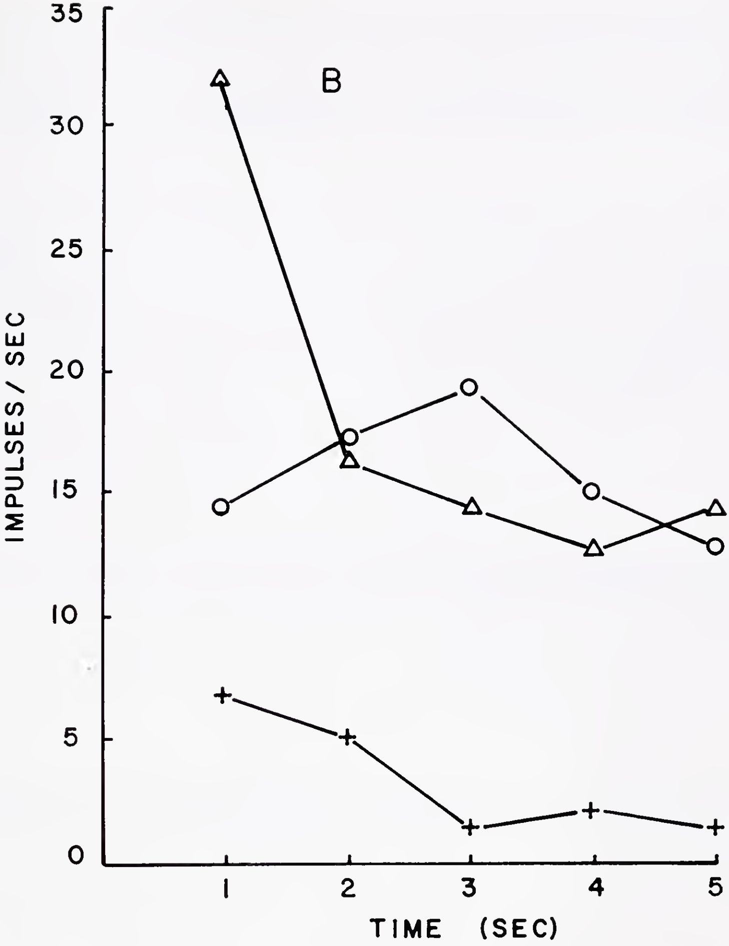
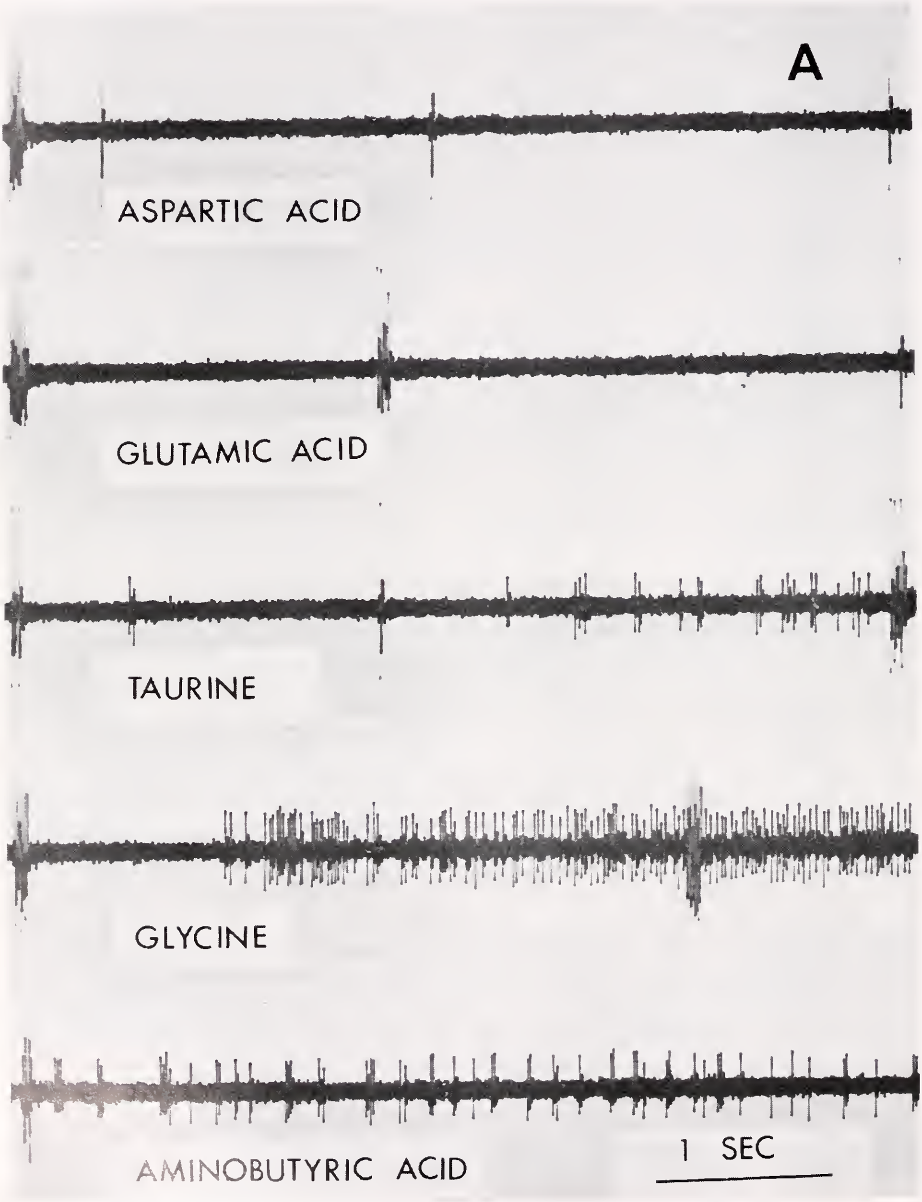


FIGURE 1. Receptor specificity in *P. interruptus* antennule. Symbols represent an averaging of responses to glycine and DL alpha amino-n-butyric acid (cross), L-aspartic acid and L-glutamic acid (open circles), and taurine (open triangles). Four preparations responded primarily to monocarboxylates (A), and seven to dicarboxylates (B). Taurine was more stimulatory in (B). Stimulus is one drop of 0.01 M of a given amino acid.

seconds; responses to 10^{-2} M lasted up to one minute. Laverack (1964) and Ache (1972) reported antennule receptor responses of similar duration to high amino acid concentrations in *Panulirus argus* and *Homarus americanus*. Response latencies were not critically examined and were often masked by mechanoreceptive activity, but in general, activity ensued within 0.1 seconds of stimulus introduction. Chemoresponses were reversible by washing the preparation with sea water.

Cancer antennarius—chemoreceptive acuity. The response threshold of both dactyl and maxilliped receptors was 5×10^{-5} M of the mixture. Response characteristics were similar to those reported by Case (1964) in an extensive study on the dactyl receptors of this species, except that responses of greater duration elicited by high concentrations were occasionally recorded. Response intensity generally peaked within one second of stimulus introduction and usually decayed rapidly in comparison to the more tonic receptor responses of *G. ingens.* Responses to one drop of 10^{-3} M of the mixture generally extinguished within 5 seconds, while re-



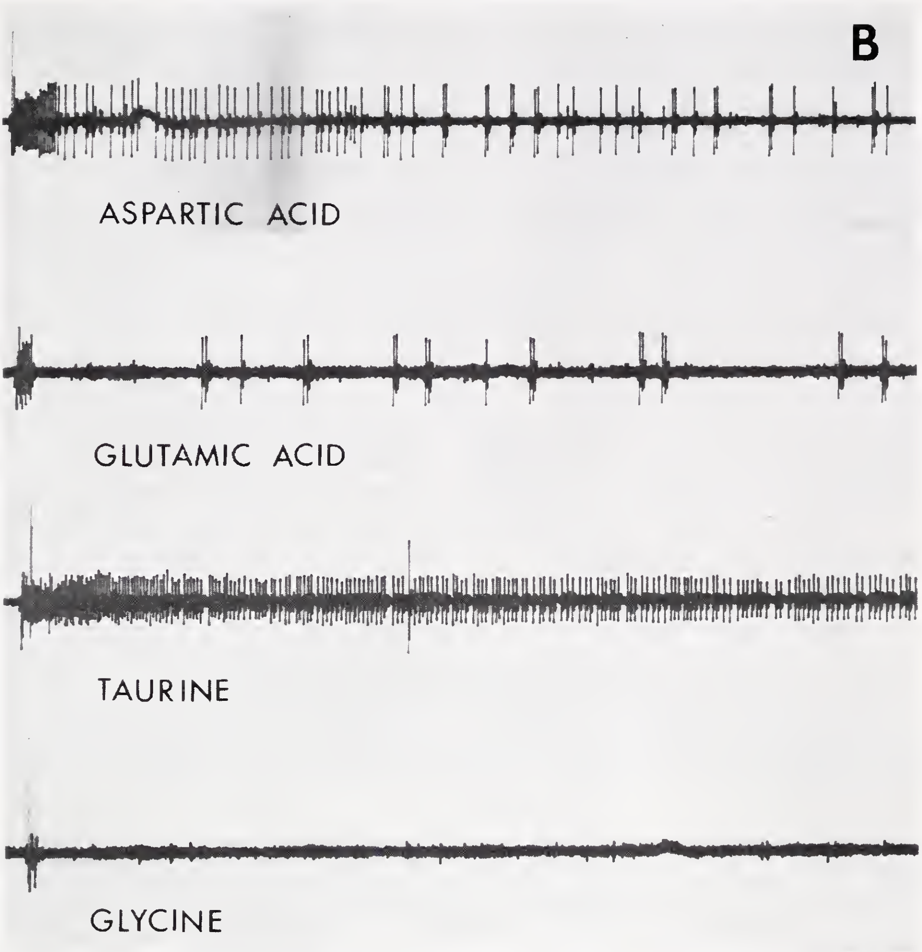


FIGURE 2. Two examples of receptor specificity in the *P. interruptus* antennule from which the preceding graphs (Fig. 1) were obtained. Responses are from receptors of the medial flagellum. (A) shows specificity to monocarboxylates, (B) to dicarboxylates. Note strong taurine response in (B). Point of stimulation indicated by initial phasic mechanoresponse.

sponses to higher concentrations persisted at lower activity levels for up to 30 seconds, though infrequently these responses to high concentrations (10^{-2} M) lasted up to two minutes. Response time and intensity increased with concentration (Fig. 4). All chemoreceptive activity was reversible by seawater washes.

DISCUSSION

The presence of amino acid receptors in crustaceans and the initiation of feeding activity by amino acids has been reported in previous studies, but the chemoreceptive acuities of crustaceans and the high degree of chemical control over their

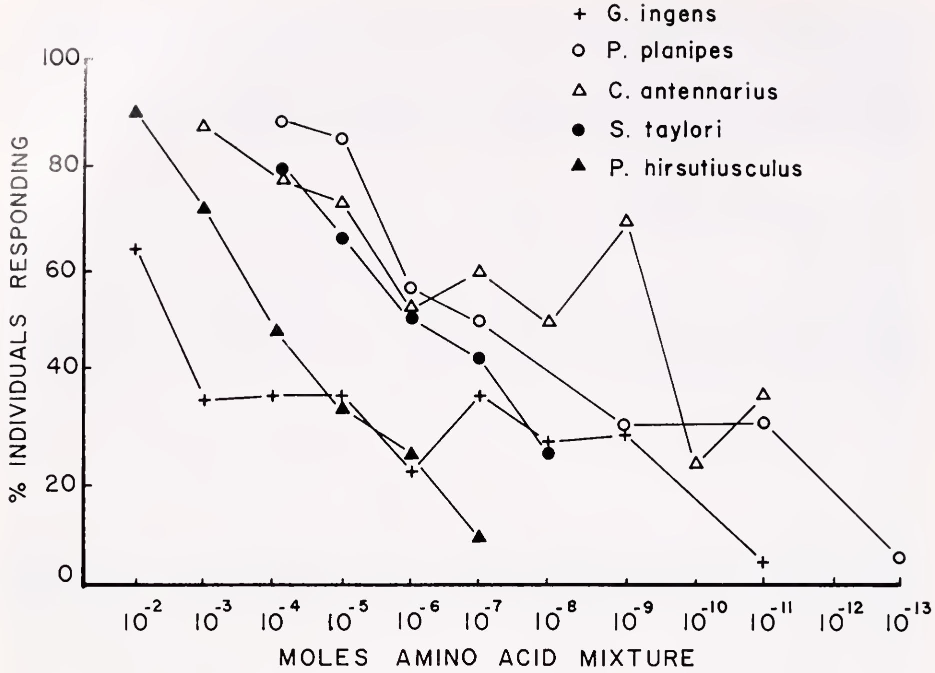


FIGURE 3. The percentage of individuals of the five species exhibiting a feeding response in the presence of a range of amino acid mixture concentrations. For each species, the percent responding to the seawater control is subtracted from the percent responding to the mixture (see Table I).

feeding behavior has not been adequately documented. This study attempts to clarify this aspect of crustacean behavior by investigating amino acid feeding response thresholds in five species from a spectrum of marine habitats. The low amino acid concentrations (10^{-7} to 10^{-12} M) at which feeding responses were exhibited show not only a higher degree of chemosensitivity than has been previously demonstrated, but also indicates that chemical cues may play a dominant role in the feeding activity of crustaceans in general, at least among the totally aquatic species. This high degree of chemical control is strikingly apparent when one observes a crab execute a capture response upon food simulated by as little as 10^{-9} M of amino acid, in the complete absence of visual cues. At higher amino acid concentrations (10^{-2} to 10^{-4} M), the feeding responses appear to occur almost as a reflex.

The greatest behavioral acuity was demonstrated by the two deeper-living species, *G. ingens* and *P. planipes,* and the predatory littoral crab, *C. antennarius.* In *P. planipes* and *C. antennarius* the amino acid mixture evoked feeding activity at minimum concentrations of 10^{-11} to 10^{-12} M, while the behavioral threshold of *G. ingens,* 10^{-10} to 10^{-11} M, was slightly higher. The two remaining intertidal species, *S. taylori* and *P. hirsutiusculus,* demonstrated thresholds of 10^{-8} to 10^{-9} M and 10^{-6} to 10^{-7} M amino acid mixture respectively. For comparison, pre-

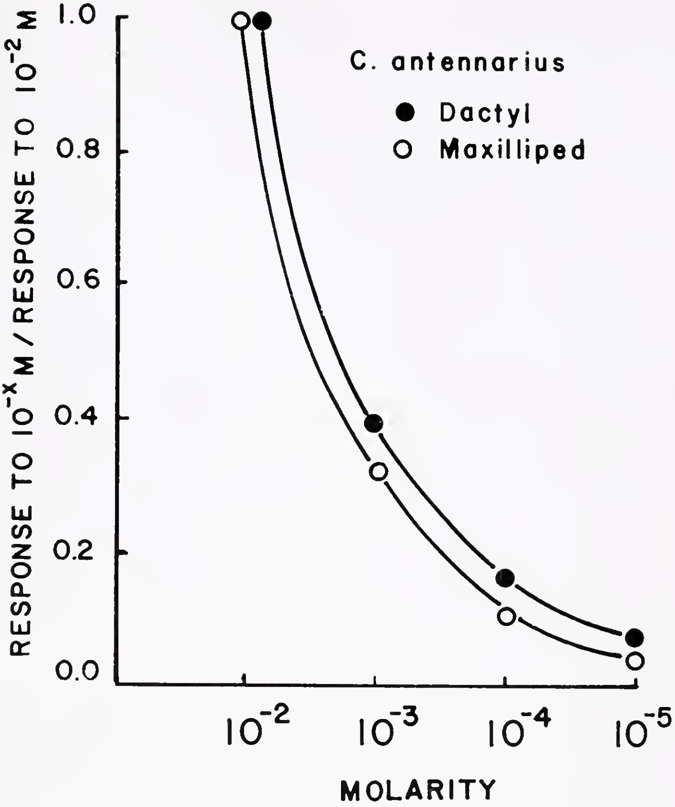


FIGURE 4. The average relative responses of 13 dactyl and 8 maxilliped preparations to a range of amino acid mixture concentrations. Receptor response to 10^{-2} M is arbitrary unity. Response intensity calculated from first 10 seconds of response.

vious behavioral studies have shown *Panulirus argus* to initiate feeding activity in the presence of 10^{-4} M amino acid (Levandowsky and Hodgson, 1965), and *Homarus americanus* to initiate food-search activity in the presence of 10^{-6} M amino acid (McLeese, 1970).

The electrophysiological thresholds for dactyl and third maxilliped receptors of *C. antennarius* were 10^{-5} M amino acid mixture. This is in agrement with the sensitivity found in the dactyl receptors of another brachyuran crab, *Carcinus maenas* (Case and Gwilliam, 1961). The dactyl receptor threshold of G. ingens (10^{-8} M amino acid mixture) is the lowest threshold reported in crustacean receptors, approximating both the antennal receptor threshold of this species (10^{-7} M amino acid mixture) and the antennular receptor threshold of 10^{-7} M amino acid reported in *Homarus americanus* (Ache, 1972; Shepheard, 1974). It has been postulated that, among the decapods, the dactyls, with lower chemosensitivity, function as contact chemoreceptors, while the more chemically sensitive antennules serve as distance receptors (Shepheard, 1974). Judging from the high chemosensitivity of the dactyl receptors of *G. ingens,* this functional dichotomy is apparently not as distinct in this bathypelagic mysid.

Similar receptor sensitivities to amino acids have been reported in fish. Recording of olfactory lobe activity in *Ictalurus catus* (Suzuki and Tucker, 1971) and *Salmo gairdneri* (Hara, 1973) showed these species possess receptors activiated by amino acid concentrations between 10^{-7} and 10^{-8} M.

The discrepancies in behavioral and electrophysiological thresholds in *G. ingens* and particularly *C. antennarius* are probably due to two factors: technical difficulties involved in isolating single receptor unit fibers, allowing the possibility that other afferent activity (mechanorceptive and proprioreceptive) masked chemoresponses at lower concentrations; and behavioral responses utilized the entire receptor population of a species, possibly including more sensitive receptors not examined electrophysiologically. In *C. antennarius,* antennule receptors were not examined electrophysiologically because of the small size and delicate nature of these appendages. Antennule receptors may have been responsible for the lower behavioral threshold observed in this species.

Another factor to be considered is that, of the species tested, *G. ingens* was the least suitable for behavioral study. This entirely pelagic species normally functions at great depths in essentially unlimited amounts of space, and is thus unaccustomed to the vertical and horizontal barriers imposed by captivity. Because this aspect of its environment was virtually impossible to simulate, this species, though an active feeder in captivity, was unable to exhibit orientation responses under test conditions. Therefore, only the most obvious feeding display (coordinated extension of the endopodites) was available as a test criterion. The unnatural environment may have served to raise the behavioral threshold of this species, particularily in light of the dactyl receptor responses to concentrations 1000 times more dilute than the *C. antennarius* dactyl threshold.

Finally, the behavioral acuities described do not represent the absolute receptor thresholds of these species, but rather are the amino acid concentrations that contained sufficient information about potential food, upon integration in the central nervous system, to stimulate the animal to feed. Furthermore, the defined feeding responses are direct attempts to secure food simulated by amino acids, and are thus conservative indices of the awareness of a chemical presence. While orientation responses, such as increased antennule flicking, also indicate the awareness of a chemical presence and occurred with greater frequency and at lower concentrations, the defined feeding responses were chosen because they provided less ambiguity.

The mixture of amino acids used in this study may have had a more stimulatory effect on feeding activity than single amino acids. *Panulirus interruptus* antennule receptors demonstrated some degree of specificity to either mono- or dicarboxylic amino acids, implying that a combination of these compounds could have an additive stimulatory effect (independent of concentration) by activating more receptor types, while single amino acids or mixtures of closely related compounds would be likely to compete for receptor sites. In an extensive study of responses to a spectrum of amino acids and other organic compounds, Shepheard (1974) similarly described two general classes of receptors in the antennules of *H. americanus* which responded maximally to either L. glutamic acid or hydroxy

L-proline. Laverack (1964) and Ache (1972) also reported amino acid specificity in crustacean antennule receptors, but, as in this study, non-specific activation was more commonly encountered, implying that either both receptor types were present in the recorded population, or that there are also receptors with broader specificity. An increased stimulatory effect of the mixture was also supported behaviorally in *P. hisutiusculus,* in which the mixture was more stimulatory than equal concentrations of the individual components (Table III). McLeese (1970), and Shelton and Mackie (1971) have also reported that crustaceans are maximally stimulated by combinations of compounds.

Another aspect of the *P. interruptus* antennule study which warrants attention is that the receptor responses to amino acids were recorded from receptors of the inner or medial flagellum of the antennule. In previous studies involving similar species (Laverack, 1964; Levandowsky and Hodgson, 1965; Ache, 1972; Shepheard, 1974) chemoreceptor activity has been reported only in the outer or lateral flagellum, which has a hair tuft encompassing aesthetasc hairs, lacking in the medial flagellum. If the medial flagellum possesses receptors activated by food odors (amino acids), it is possible the elaborate hair tuft of the lateral flagellum has a more specialized function, such as pheromone reception, in addition to amino acid reception.

Possible mechanisms involved in orientation to chemical cues may be inferred from the results of this study in conjunction with the work of previous investigators. We wish to briefly discuss four which warrant further investigation: quantitative discrimination, qualitative discriminaton, contact between chemoreceptive appendages in general, and contact between thoracic appendages and mouthparts.

The direct relationship between stimulus concentration and receptor response intensity, also noted by Case (1964) and Ache (1972), would potentially provide the ability to orient up a chemical gradient in a food search. The apparent discrimination of various amino acids would afford qualitative analysis of chemical feeding stimulants and support Shepheard's (1974) contention that crustaceans may distinguish these compounds from one another.

Contact between chemoreceptive appendages (brushing or combing one appendage with another) following the introduction of chemical stimulants was noted in the five species examined behaviorally. This "preening" activity occurred in such forms as rubbing maxillipeds together, rubbing periopodites together, or combing antennae or antennules with maxillipeds, periopodites or chelipeds. Because this activity occurs primarily among chemoreceptive appendages following reception of chemical cues, and because it appears to be divorced from direct procurement of food, it possibly serves orientation through enhancement of chemical reception. While this activity may simply represent a mechanical cleaning of receptor sites, unpublished results found by Lindsey (1975, University of California) indicate the mechanoreceptive activity, as would be induced by contact between appendages, may actually influence chemoreceptor activity at the neuronal level.

A more specific form of appendage contact with perhaps more obvious implications is the action of placing the chelipeds to the mouth, as noted in three test species. This may simply represent direct attempts to ingest food simulated by amino acids. However, the particularily habitual nature of this activity in *P.*

hirsutiusculus, which executes this activity with rapidity and regularity when chemically stimulated, suggests this behavior may serve to present potential food or food odors to receptors at the mouth for verification of proper chemical cues before actual feeding is initiated. This mechanism has not been studied in crustaceans, but Atema (1971) reported that in catfish (*Ictalurus natilis*) deinnervation of chemoreceptors on the mouth blocked acceptance of food, even though intact external receptors enabled the fish to locate the food. Perhaps a similar dichotomy of function exists in crustaceans.

Focusing on *G. ingens* and the utilization of high chemosensitivity in the bathypelagic environment, Vinogradov (1970) has suggested that the two primary food sources of species below depths of 500 meters would be sinking zooplankton remains and vertical migrators transporting the resources of the more productive surface waters downward to non-migrators, such as *G. ingens.* This implies that deeper-living pelagic species would be scavengers or filter feeders of zooplankton remains and/or carnivores preying directly or indirectly upon vertical migrators. Calman (1909) suggested that *G. ingens* is a coarse filter feeder based on the existence of "feeding currents" produced by the maxillipeds. Our observation of the modification of the hairs of the anterior endopodites into sieve-like structures and the circular motion of these appendages over the mouth in the presence of amino acids supports this hypothesis. However, such factors as gut contents, fecal material, rapacious feeding upon fish caught in the same trawl, and apparent capacity through size and mobility (it is the largest entirely pelagic crustacean) indicate that *G. ingens* preys upon crustaceans and small fish of the midwater. This species may therefore function both as a scavenger and predator, consuming any usable organic material it encounters.

The high chemoreceptive abilities and the hypothesized mechanism of orienting up a chemical gradient may serve to detect prey through leakage of these compounds, possibly facilitated by tissue damage or through normal waste excretion. A particularly high percentage of individuals responded to betaine (Table II), a methylated amino acid form associated with protein synthesis and breakdown, found in the blood and excretory products of both vertebrates and invertebrates (Kutscher and Ackerman, 1933). Betaine would also be expected in decomposing zooplankton. The high frequency of feeding response exhibition to tactile stimulation in the absence of amino acids (48%) indicates mechanoreception is also an important feeding cue. In the absence of light, a food search initiated by dilute chemical cues would be effectively executed by the antennae, which are longer than the body and extend laterally and back, and by the antennular rami, one of which extends forward, the other laterally and ventrally. These appendages would thus monitor three axes of the immediate search area.

In conclusion, the bathypelagic mysid *G. ingens* is among the more chemosensitive of the species examined, and this capacity is probably of primary importance in locating food in an essentially aphotic environment. However, we have observed that intertidal species also exhibit feeding behavior in the presence of low amino acid concentrations in the absence of visual cues. It is likely there is a high degree of chemical control over the feeding behavior of aquatic crustaceans in general. Visual cues appear to generally play a conparatively minor role, even in the shallower-living species, so the absence of light may not pose as significant an orientation problem to bathypelagic crustaceans as might be assumed.

We wish to thank Dr. James F. Case for his advice and the use of equipment essential to the electrophysiological aspects of this study. We also thank Jose Torres, Langdon Quetin, Kathy Hamilton and David Cook for critically reviewing the manuscript.

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SUMMARY

1. Feeding response exhibition in five marine crustaceans was observed in the presence of a range of concentrations of an amino acid mixture composed of equal parts DL alpha amino-n-butyric acid, L-glutamic acid and taurine. *Gnathophausia ingens, Pleuroncodes planipes,* and *Cancer antennarius* displayed behavioral thresholds of between 10^{-10} and 10^{-12} M; *Spirontocaris taylori* and *Pagurus hirsutiusculus* displayed thresholds between 10^{-7} and 10^{-9} M.

2. Electrophysiological response thresholds to the amino acid mixture were examined in *G. ingens* and *C. antennarius.* The antennular and dactyl receptors of *G. ingens* displayed thresholds of 10^{-7} and 10^{-8} M respectively. Maxilliped and dactyl receptors of *C. antennarius* both displayed a threshold of 5×10^{-5} M.

3. *Panulirus interruptus* antennule receptors displayed a degree of specificity to mono- and dicarboxylic amino acids.

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STUDIES ON THE LIFE HISTORY OF TWO CORAL-EATING NUDIBRANCHS OF THE GENUS *PHESTILLA*

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This report describes the results of studies on the life history of two tropical coral-eating nudibranchs of the aeolid genus *Phestilla*. *Phestilla melanobranchia* Bergh, 1874, feeds primarily on ahermatypic corals in the Family Dendrophylliidae; *Phestilla sibogae* Bergh, 1905, feeds on hermatypic corals in the Genus *Porites*. Both species were reared through their entire life cycle in the laboratory.

Adult morphology is strongly emphasized in the taxonomic literature on nudibranchs but little information is available about such aspects as egg mass and veliger morphology (Hurst, 1967), factors influencing metamorphosis (Harris, 1973), growth and fecundity rates and longevity (Miller, 1962 and Thompson, 1964). The egg mass of *Phestilla sibogae* was described by Kawaguti (1943) and Ostergaard (1950). Bonar (1973) and Bonar and Hadfield (1974) have given detailed descriptions of morphological and ultrastructural changes during metamorphosis in *Phestilla sibogae.*

Attempts to cultivate different developmental types have been summarized by Hadfield (1963), Thompson (1967) and Harris (1973). To date only two nudibranch species with a planktotrophic veliger stage have been reared through metamorphosis. K. Engel (personal communication, 1971, University of California at Santa Barbara) raised the aeolid *Hermissenda crassicornis,* and the development of *Phestilla melanobranchia* is described below.

Providing the factor or factors necessary to induce metamorphosis is a key criterion for the successful cultivation of nudibranchs. The factors influencing metamorphosis fall into two categories (Harris, 1973). In all cases reported, a host factor or chemical cue has proved necessary. Some nudibranch species require only this chemical cue (Thompson, 1958; Hadfield and Karlson, 1969), while others also require contact with the prey species (Thompson, 1962; Tardy, 1962b). The prey substance initiating metamorphosis appears to be proteinaceous (Tardy, 1962b) and may be associated with the mucus of the prey (Hadfield and Karlson, 1969).

Two general nudibranch life cycle groupings have been proposed by Miller (1962) and Thompson (1964). The first contains nudibranchs which are short-lived, seasonal and feed on fast-growing, seasonal prey such as hydroids (Rasmussen, 1944; Swennen, 1961; and Tardy, 1962a and b). The second group is long-lived with annul life cycles and tends to feed on slow-growing, long-lived prey such as anthozoans and sponges (Thompson, 1958, 1961b and 1962; Swennen, 1961 and Potts, 1970).

Materials and Methods

All stages of the life cycle of *Phestilla melanobranchia* and *P. sibogae* were observed in the laboratory. Rearing of the veliger stage was attempted only at the Hawaii Institute of Marine Biology, while observations on adult stages were made both in Hawaii (October 1966 to June 1967 and October 1968 to March 1969) and at the University of Singapore (September 1967 to June 1968).

Embryology of Phestilla melanobranchia

Mature specimens of both species of *Phestilla* deposit one or two egg masses per day for up to three months. To observe the embryology of *Phestilla melanobranchia* ten egg masses were isolated within an hour of deposition and maintained at 22° C in filtered sea water changed daily.

Small pieces of each egg mass were isolated in depression slides and observed at regular hourly intervals for the first three days, and then once or twice daily thereafter until all egg masses had begun to hatch. The water in the depression slides was changed every two to five hours, and new pieces of egg mass were used in each 24-hour observation period. After each new stage of development, a piece of corresponding egg ribbon was cut off and preserved in FAA (formalin, ethanol and glacial acetic acid in sea water).

Veliger cultivation

The techniques used for culturing veligers were adapted from those of Loosanoff and Davis (1963), and Struhsaker and Costlow (1968). Egg masses were placed in 10 cm diameter stacking dishes, and veligers were allowed either to hatch naturally or egg masses were artificially opened. All manipulation of veligers was accomplished by use of breath-controlled, finely-drawn glass pipettes. Transfer of the photospositive veligers was done in a darkened room with the beaker on a black background and a light source on one side. The veligers congregated on the lighted side where they could be collected and counted easily.

Veligers were cultured in 250 ml beakers containing 200 ml of water. The water was filtered through a styrofoam "Cuno" filter and changed daily.

Two cultured algae were used as food sources. The diatom *Phaeodactylum tricornutum* was used in all experiments and the green flagellate *Dunaliella* sp. was combined with *P. tricornutum* in a few cases. Five to 15 ml of algal culture were added daily.

The antibiotic penicillin was tested in several experiments to suppress bacterial growth in the cultures. The concentration of penicillin was 0.5–1.0 units/ml of culture medium.

Two techniques prevented the photopositive veligers from being trapped in the surface tension. In all experiments dark covers were placed over the upper half of the beakers so that light did not come from above. In later experiments, a few small cetyl alcohol flakes were sprinkled on the surface to reduce the surface tension, making the veliger shells less hydrophobic (Hurst, 1967).

In the first series of experiments (February to June 1967) temperatures of 25 to 27° C proved to be the most effective for veliger maintenance, but during the second series (October 1968 to February 1969), available laboratory facilities necessitated culturing at 21° C.

Small pieces of coral were added to the culture beakers to induce metamorphosis of the veligers. Small whole polyps of *Tubastraea aurea* were initially used for

Phestilla melanobranchia veligers, but pieces of skeleton and tissue taken from polyps of *Dendrophyllia elegans* were more effective and were used in most experiments. Small pieces of *Porites* colonies were used to induce metamorphosis in *Phestilla sibogae* veligers. I found that specimens of both *P. melanobranchia* and *P. sibogae* were best left undisturbed once settling had begun and so did not change the water in the beakers for several days after introducing the coral. When all veligers had completed metamorphosis, they were transferred with the original pieces of coral into 10 cm diameter stacking dishes. When the nudibranchs were 2–5 mm long they were moved to one of the systems used for adult animals.

Maintenance of adult nudibranchs

The two species of *Phestilla* were kept in the laboratory by three separate methods: running seawater tables, aerated aquaria and individual bowls. In Hawaii, nudibranchs were left in running seawater tables in which corals were also kept. All of the species of ahermatypic dendrophylliid corals found in Hawaii, as well as hermatypic corals in the genera *Porites, Montipora, Fungia, Pocillipora, Cyphaestrea, Pavona* and *Leptastrea,* were available for nudibranchs to feed on.

A running seawater system was not available in Singapore, so the corals used to feed *Phestilla melanobranchia* were kept in 20 l aquaria.

Nudibranchs were also maintained separately or in pairs in plastic dishes approximately 20 cm in diameter and 10 cm in height. The water was changed daily, and the nudibranchs were supplied with small colonies of coral for food. By keeping individual animals isolated for long periods of time, it was possible to collect information on different aspects of their biology such as life span, growth rates, fecundity, color changes, preferences for coral species and many behavioral patterns.

RESULTS

Sexually mature specimens of *Phestilla melanobranchia* or *P. sibogae* maintained with fresh coral and sea water laid an average of 1.5 egg masses per day for up to 100 days. The egg mass of each species is a flattened ribbon attached in a circle along one edge so that it flares somewhat. The eggs are arranged in tightly folded rows, oriented perpendicularly to the long axis of the ribbon. There is only one egg per capsule and the dimensions for egg mass, capsule and egg for both species of *Phestilla* are given in Table I. Egg masses for *P. melanobranchia* contain from 1,000 to 4,000 eggs each, with an average of about 3,000. Eggs of *Phestilla sibogae* are slightly larger than those of *P. melanobranchia,* and the egg masses contain fewer eggs, usually less than 3,000.

TABLE I

Sizes of egg masses, eggs, and capsules, veliger shells and newly metamorphosed stage in millimeters.

Species	Egg mass		Mean egg		Mean egg diameter	Mean veliger shell length	Newly metamorphosed stage mean length
	Length	Width	Length	Width			
P. melanobranchia	12–25	4–6	0.23	0.148	0.115	0.20	0.225
P. sibogae	15–25	4–7	0.28	0.18	0.148	0.264	0.30

Phestilla melanobranchia

The embryonic development of *Phestilla melanobranchia* is similar to that described for the aeolid *Fiona marina* (Casteel, 1904) and *Aeolis* (= *Cuthona*) *concinna* (Pelseneer, 1911).

The landmark stages in the embryonic development of *P. melanobranchia* and the average cumulative times for 10 egg masses at 22° C are as follows: deposition—0 hours, appearance of polar bodies—1 hour, 2-cell stage—4 hours, 4-cell stage—5 hours, 8-cell stage—6 hours, 12-cell stage—7 hours, 16-cell stage—8 hours, blastula—16 hours, gastrulation—29–40 hours, first movement—88 hours, torsion—112 hours, hatching—136–160 hours.

The cleavage rate varied among egg masses, but within a single egg mass the early cleavages appeared to be synchronized. Pieces of egg mass removed from the main mass often had cleavage rates out of phase with the main mass, but within the removed pieces cleavages remained synchronous.

Torsion in *Phestilla* entails a distinct 180 degree turning of the head and foot with respect to the shell. The shell is oblong and inflated and corresponds to Type 2 described by Thompson (1961a). The veligers are ready to hatch about 20 hours after torsion or about five days after deposition.

Veligers normally rotate slowly in the egg capsules which are surrounded by a gelatinous matrix. Only when the matrix disintegrated and the egg capsules came directly in contact with sea water did the veligers become active; then they would escape in one to two minutes. Veligers were repeatedly observed grasping the thin, flexible capsule wall with the mouth. Shortly thereafter, the veligers would escape through a hole in the capsule wall which corresponded to the area contacted by the mouth. Hatching veligers do not have a radula.

External factors are important in breaking down the matrix of the egg mass. The matrix of egg masses collected immediately after deposition and kept in motionless filtered sea water did not break down. Egg masses collected in the field, or left in running sea water tables for several days, broke down; they were subjected to water movement and colonization by bacteria, protozoans, nematodes, annelids and crustaceans. The fauna commonly associated with egg masses was not observed to attack developing veligers.

Phestilla melanobranchia veligers have little tissue inside the shell at hatching. They were very active and positively phototactic. Healthy, feeding *P. melanobranchia* veligers grew slowly until by the seventh day the body filled about two-thirds of the shell. The digestive gland was dark brownish-green from the *Phaeodactylum* pigments. No growth of the shell was observed after hatching. During this period, the foot thickened and by the eighth day, a propodium had formed and the veligers could crawl. When the veligers were ready to settle, they responded less to light, slowed their movement, and spent most of their time on the bottom. The velum appeared smaller and the color of the digestive gland faded, indicating a decrease in feeding.

Table II summarizes the results of culture experiments. In the one cultivation attempt where 13% of the veligers survived through metamorphosis, a motile green flagellate, *Dunaliella* species, was used in combination with *P. tricornutum*. Unfortunately, the *Dunaliella* cultures became contaminated with *Phaeodactylum* and were discarded thereby prohibiting further use of the alga.

TABLE II

Veliger cultivation results. Except where stated, the food source for veligers was the alga Phaeodactylum tricornutum.

Expt. number Date	Number beakers	Initial veliger	Veligers crawling	Metamorphosed	Percent metamorphosed	Coral species
Phestilla melanobranchia (Experiments 1–15 were unsuccessful and values given are average except where stated)						
Expt. 1–15	7	1403	0–1+[1]	0	0	*Tubastrea aurea* (expt. 1–4) *Dendrophyllia elegans* (expt. 5–15)
Expt. 16	2	400	4+	4	1.0	*D. elegans*
Expt. 17	2	400	0	0	0	*D. elegans*
Expt. 18[2]	2	800	many	107	13.4	*D. elegans*
Expt. 19	2	750	0	0	0	*D. elegans*
Expt. 20	2	1500	0	0	0	*D. elegans*
Phestilla sibogae						
Expt. 1 28 Oct. '68	1	125	many	67	53.6	*Porites compressa*
Expt. 2 30 Nov. '68	1	200	30	30	15.0	*P. compressa*
Expt. 3 7 Jan. '69	1	200	many	60	30.0	*P. compressa*
Expt. 4a	1	315	many	124	39.4	*P. compressa*
Expt. 4b 17 Feb. '69	1	350	many	12	3.4	*P. compressa*[3]

[1] One or more crawling veligers seen in eight out of 15 experiments.
[2] The alga *Duneliella* was used in combination with *P. tricornutum.*
[3] *Porites compressa* skeleton for two days and then live *P. compressa.*

Cetyl alcohol was very effective in keeping the shells of the veligers from becoming trapped in the surface film. When no cetyl alcohol was used, up to 100 out of 300 veligers might become caught in the surface tension between changes, whereas less than 25 would be trapped when it was used. Trapped veligers invariably accumulated material on their shells and tended to become attached to the bacterial film on the bottom where they were susceptible to bacterial and ciliate invasion.

Veliger density affected survival. Mortality was less at the concentration of 150 to 200 veligers per 100 ml than at 50 to 100 veligers. Bacterial activity seemed to be suppressed in the more crowded cultures.

Living coral tissue triggers settling and metamorphosis in *P. melanobranchia.* Small pieces of living *Dendrophyllia elegans* polyps were particularly effective for *P. melanobranchia.* Veligers could not settle directly on the tissue, and they were eaten if they encountered the tentacles of a whole polyp. Therefore, small pieces of skeleton and tissue were used. Such coral fragments lived for long periods if the water was changed regularly.

Once the *P. melanobranchia* veligers had settled on the skeleton near the tissue, the velum regressed quickly and disappeared in about 24 hours. The digestive gland lost all color and the body came free of the shell within 24 to 48 hours. The small nudibranchs became vermiform in shape within 24 hours after dropping their shells. Feeding began shortly thereafter and was indicated by the yellow and red coloration of the digestive gland. The buds of the first cerata also appeared at this time.

In *P. melanobranchia*, the most critical period for successful cultivation was that following the addition of coral. Crawling veligers were observed in eight of the first 15 culture attempts with *P. melanobranchia,* but in each case attempts to isolate these anmials were either unsuccessful or the experiment was terminated too soon after the coral was added. Had the cultures been left alone after the addition of coral, successful metamorphosis would likely have been a far more regular occurrence.

The results of growth rate measurements are summarized in Table III. The percent increase in body length was greatest in small animals and dropped off with size. There was a distinct drop in growth rate at a length of about 20 mm, which corresponded to the onset of egg mass production. The maximum length attained in the laboratory was approximately 40 mm; specimens of comparable length were collected in the field. *P. melanobranchia* was recorded from egg to egg in 60 days and the maximum life span was over 140 days.

In *P. melanobranchia* the male system is functional by the time the nudibranchs reach 10 mm in length. The female system becomes functional when they reach 20 mm as indicated by egg mass production. If a nudibranch has mated with another animal 10 mm or larger, then the eggs are fertile and they will develop. If the nudibranch has not mated, no development takes place. Egg masses from mated nudibranchs are 100% fertilized. Specimens of *P. melanobranchia* receive enough sperm from a single mating to last for more than two weeks of continuous laying.

A summary of egg mass production is presented in Table IV. Egg mass production was continuous for the greater part of the life span with an average

Table III

Summary of growth rate data in millimeters per day and percent increase per day, from Harris, 1973.

	0.2*–5 mm	5–10 mm	10–15 mm	15–20 mm	20–25 mm	25–30 + mm
Phestilla melanobranchia						
Hawaii						
Average	0.34	0.50	0.86	1.27	1.00	0.79
% Increase	12.25	6.27	6.41	6.76	4.27	2.79
Singapore						
Average	0.27	0.41	0.82	0.62	0.73	0.62
% Increase	9.78	5.13	6.15	3.39	3.03	2.20
Phestilla sibogae						
Hawaii						
Generations F_{1-3}						
Average	0.46	0.62	0.94	1.30	0.83	0.69
% Increase	15.47	7.64	7.60	6.91	3.54	2.44

* Length immediately after metamorphosis.

TABLE IV

Fecundity and life span/data.

	Number nudibranchs in sample	Mean maximum number egg masses per day	Mean number egg masses per day	Mean minimum number egg masses per day	Maximum total egg masses (number days)	Life span in laboratory
Phestilla melanobranchia						
Singapore	41	2.72	1.82	0.16	140 (76)	86 days 17 mm*
Hawaii	48	2.63	1.53	0.76	152 (118)	135 days 8 mm*
Phestilla sibogae						
Hawaii Field	3	1.55	1.52	1.50	175 (117)	122 days**
Hawaii Lab. F_1	10	1.94	1.57	1.14	178.5 (114)	139 days†
Hawaii Lab. F_2	8	1.79	1.45	1.00	113 (83)	114 days†
Hawaii Lab. F_3	10	2.06	1.71	1.31	101 (53)	84 days**†
Hawaii Lab. F_4	10	2.33	2.08	1.70	22 (10)	43 days**†

* = nudibranch size when collected.
** = terminated early.
† = from hatching as veliger.

of more than 1.5 egg masses being laid each day; this was consistent as long as adequate food and clean sea water was provided. Young individuals produced an average of nearly two egg masses per day and the average daily production decreased as the animals grew older.

Removal of food stimulated an increased egg mass production for a few days and return of coral caused cessation of egg mass laying for a day or two before normal production resumed.

Phestilla sibogae

Development of *P. sibogae* to the veliger stage is identical to that of *P. melanobranchia.*

Hatching *P. sibogae* veligers, which are lecithotrophic, look very much like *P. melanobranchia* veligers that have been feeding for seven days; the shell is filled with tissue though the digestive gland has no color. *P. sibogae* veligers were inactive for two days and spent much time on the bottom. They fed little or not at all, and the digestive gland did not turn dark. By the third day, the veligers had developed a propodium on the foot and crawled and swam alternately. They were markedly more active than on the two preceding days, and swam quickly toward a light source placed to one side of the beaker.

Living *Porites* tissue added on the third day induces *P. sibogae* veligers to metamorphose within 24 hours (see Table II). *P. sibogae* settles directly on

Porites tissue as well as anywhere in the beaker, as long as coral is present. One *P. sibogae* veliger was observed to metamorphose in the absence of any coral tissue, but this was one out of 300 veligers.

Both species settled at relatively specific times, three to four days after hatching for *P. sibogae* and eight to ten days for *P. melanobranchia*. *P. sibogae* veligers began to die if they did not hatch in eight days, and five days following hatching the majority of the veligers would not respond to live *Porites* tissue.

After metamorphosis, *P. sibogae* had a higher percent increase in body length than did *P. melanobranchia* and the growth rate slowed in both species at a body length of about 20 mm which corresponds to the onset of egg mass production (see Table III). Both *Phestilla* species attained a maximum length of approximately 40 mm in the laboratory.

The fecundity of *P. sibogae* was similar to that of *P. melanobranchia,* with individuals of both species producing about one and a half egg masses per day (see Table IV). A comparison of the *P. sibogae* F_1 and F_2 generation production data with that of the prematurely terminated F_3 and F_4 generations clearly demonstrates the decrease in egg mass production with time.

A faster growth rate shown by *P. sibogae* combined with the shorter period from hatching to metamorphosis explain the differences in generation time between the two species. *P. melanobranchia* was cycled from egg to egg in 60 days, while *P. sibogae* averaged 38 days over four generations. The maximum life span for each *Phestilla* species was approximately 140 days, though *P. melanobranchia* may exceed this by two or three weeks.

P. melanobranchia was more sensitive to crowding and water fouling than *P. sibogae*. *P. melanobranchia* maintained alone or paired on *Tubastraea aurea* or *Dendrophyllia elegans* generally did very well. Survival of *P. melanobranchia* in groups of more than two was markedly reduced. *P. sibogae* was much hardier under crowded conditions and closely packed aggregations of over 100 adults were common in the running seawater table where a large population was maintained.

The water from Kaneohe Bay, Hawaii, had a high organic content, and on several occasions, the water became foul in less than 24 hours. *P. sibogae* proved to be quite resistant to this fouling, but *P. melanobranchia* was not able to tolerate it and twice most of the *P. melanobranchia* being maintained in bowls were killed.

Discussion

Serial culture of an animal in the laboratory opens a broad vista of studies for which it may be utilized. *Phestilla sibogae,* whose initial cultivation in 1969 is described here, is still under cultivation (Hadfield and Karlson, 1969; Bonar, 1973; Bonar and Hadfield, 1974). Also, knowledge of the complete life cycle of an animal provides important insights into its biology. While the life histories of the two *Phestilla* species are similar, they show differences which can be linked to the biology of their respective coral prey. The differences between the two species, and from other aeolids reported in the literature, will be discussed below.

The embryology of *Phestilla* follows the general pattern reported for other nudibranch species (Casteel, 1904; Pelseneer, 1911). The complete development of *Phestilla* from newly laid egg to hatching veliger takes five to seven days at 21–25° C. Developmental times of a number of aeolid species reported in the literature are similar to this (Hadfield, 1963).

Observations on the embryology of *P. melanobranchia* suggest that some mechanism synchronizes early cleavage within an egg mass. Such a mechanism could act as a pace setter which would insure completion of development for most of the veligers before the egg mass begins to deteriorate. It also suggests possible chemical mediation within the egg mass.

The consistent observation that veligers are dependent on external factors to erode the gelatinous matrix of the egg mass is surprising. Observations on the egg mass of other species suggest that this may be a general phenomenon among nudibranchs. Important factors in this breakdown appear to be water movement, bacterial degradation and burrowing by small animals such as nematodes and annelids.

An important problem in rearing the veligers of *P. melanobranchia* was keeping the food supply in suspension. Shaker tables were tried but veliger entrapment by surface tensions made this method impossible. A constant food source would increase the growth rate, perhaps resulting in a reduced cultivation time, and increased survival. The highest survival rates for *P. melanobranchia* occurred when the motile alga *Dunaliella* sp. was used in combination with *Phaeodactylum*. Unfortunately the *Dunaliella* cultures were lost before it was possible to exploit them further.

Living coral tissue is necessary to induce settling and metamorphosis in both species of *Phestilla*. Hadfield and Karlson (1969) found that some proteinaceous factor in *Porites* mucus is responsible for inducing metamorphosis in *P. sibogae*. A similar mechanism is likely to be responsible for metamorphosis in *P. melanobranchia*.

P. melanobranchia veligers were observed to undergo metamorphosis only in close proximity to the coral tissue, though not on it. *P. sibogae* veligers underwent metamorphosis anywhere in the beaker if coral was present. Therefore, while a chemical cue from living prey is necessary for inducement of settling and metamorphosis in nudibranchs, actual contact with the prey is not a consistent requirement (Harris, 1973).

The settling-related behavior of *Phestilla* veligers correlates well with the ecology of their respective coral prey. *Porites* corals are large, photopositively distributed and extremely common in Hawaii. Veligers of *P. sibogae* become positively phototactic when ready to settle and will settle on or in the vicinity of the coral tissue. Dendrophylliid corals are photonegatively distributed, small and patchy in distribution. Veligers of *P. melanobranchia* become negatively phototactic when ready to settle and must be in close proximity to the coral. Dendrophylliid corals will eat *P. melanobranchia* veligers, and the veligers can only settle adjacent to the tissue of the coral.

After hatching, *P. sibogae* grows faster than *P. melanobranchia* at each stage up to the adult size of about 40 mm. *P. sibogae* is ready to settle in three days and completes metamorphosis in one day more, while *P. melanobranchia* takes eight and three days respectively. After metamorphosis, *P. sibogae* reaches sexual maturity approximately two weeks sooner than *P. melanobranchia* and the maximum life spans of over four months are similar. A possible explanation for the difference in growth rate may be nutritional adaptation to the prey. *Phestilla sibogae* may utilize *Porites* tissue more effectively than *P. melanobranchia* utilizes dendrophylliid tissues. *Porites* contains zooxanthellae which *P. sibogae* digests. In addition, zooxanthellae continue to photosynthesize for several days in the digestive gland of *P. sibogae* (L. Muscatine, personal communication, 1969, University of California, Los Angeles). The role of zooxanthellae in the nutrition of *P. sibogae* and the comparative energetics of these two nudibranch associations would be rewarding areas of further study.

Both *Phestilla* species begin laying egg masses when they are about 20 mm in length and continue to spawn and feed for about 100 days. There is no interruption of feeding such as has been found in some seasonal species on the coast of England. Thompson (1958, 1961b) reported that several dorid species stop feeding when they begin spawning and actually starve to death before they deplete their reproductive capabilities. Most aeolids described in the literature and observed by the author continue to feed and spawn for considerable periods (Rasmussen, 1944; Tardy, 1962a and b). Individuals of both species of *Phestilla* survived for more than a week after they ceased laying and the gonads were depleted; during this time they fed and continued to associate closely with their mates.

Several generations of each *Phestilla* species are produced in a year, placing them in Miller's (1962) Group 2. The numbers of *Phestilla melanobranchia* did not vary greatly during different times of the year, and the number of nudibranchs in a given area depended on the concentration of dendrophylliid corals available. The species of nudibranchs listed by Miller (1962) and Thompson (1964), which have several generations a year, generally feed on fast growing, seasonal prey. The prey of both species of *Phestilla* is stable and slow growing, so they might be expected to live longer than the observed maximum of four and a half months. *Phestilla* is a member of the Family Cuthonidae, other species of which are small, fast-growing hydroid eaters; thus the growth rates of the *Phestilla* species may be more influenced by their genetic background than the stability of the food source.

Predation on nudibranchs may be another factor influencing longevity. To grow fast and reproduce as rapidly as possible before being eaten may be far more important than the availability of the food source. Differences between generation time of the two species of *Phestilla* may also be influenced by predation pressures.

P. sibogae is more susceptible to predation than *P. melanobranchia* and this pressure could in time have selected for faster growth. Pressure from predation combined with the great abundance of *Porites* could have led to lecithotropic development thereby minimizing exposure of the veliger stage to predation (Thorson, 1946). In contrast, the patchy distribution of dendrophylliid corals could tend to select for a longer veliger stage thereby providing more dispersal time in *P. melanobranchia*.

In conclusion, the differences in life cycles of *P. melanobranchia* and *P. sibogae* can be related to the biology of corals. Harris (1970) also found that pigmentation, morphology and adult behavior all reflect characteristics of the prey. The differences in generation time between *Phestilla* and other nudibranchs with stable prey populations is likely to be related to adaptations to avoid predation and/or the affinities of *Phestilla* with the Family Cuthonidae.

It is the author's belief that many nudibranch species could be cultivated successfully if one were willing to devote the time necessary to work with the veliger stage and the prey species. Knowledge of the biology of the prey greatly facilitates the study of any nudibranch species.

A number of individuals contributed to the success of this study. Special gratitude is due my wife, Annmarie, for her encouragement and assistance in all phases of the study. I wish to also thank Cadet Hand, Emery Swan, M. Patricia Morse, Ruth Turner, Claudia Foret and Langley Wood for their constructive suggestions during the preparation of the manuscript. The study was supported by an East-West Center Fellowship through the University of Hawaii and by an Edwin Pauley Fellowship through the University of California.

SUMMARY

1. The complete life cycles of two coral-eating aeolid nudibranchs, *Phestilla melanobranchia* Bergh, 1874 and *Phestilla sibogae* Bergh, 1905, are described. Information on their life histories includes developmental stages and timing, duration of the veliger stage, veliger behavior, factors necessary for settling and metamorphosis, and adult growth rates, fecundity and longevity. The life cycles of the two *Phestilla* are similar and their physical and behavioral differences are related to the characteristics of their respective coral prey.

2. *P. melanobranchia* has planktotrophic development, is negatively phototactic when ready to settle, requires close proximity to living dendrophylliid coral tissue for metamorphosis and has a generation time from egg to egg of 60 days. The dendrophylliid corals on which *P. melanobranchia* feeds are small, patchy and photonegative in distribution.

3. *P. sibogae* which has now been under serial cultivation for four years, has lecithotrophic development, is positively phototactic when ready to settle, requires only a chemical factor from living *Porites* tissue for metamorphosing and has a generation time of 38 days. The *Porites* corals which *P. sibogae* feeds on are large, very common and photopositive in distribution.

4. Differences in predation pressure and prey tissue utilization efficiency are proposed as factors influencing the evolution of a significantly faster generation time in *Phestilla sibogae* than in the closely related *P. melanobranchia*.

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THE ULTRASTRUCTURE OF THE AFLAGELLATE SPERMATOZOON OF THE FRESHWATER TURBELLARIAN *HYDROLIMAX GRISEA* (PLATYHELMINTHES: PLAGIOSTOMIDAE)[1, 2]

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The spermatozoön of *Hydrolimax grisea* was described by Hyman (1938; p. 16) as ". . . of the type characteristic of the Plagiostomidae, with a central long filamentous nucleus and winglike lateral protoplasmic expansions." An illustration and description of staining characteristics of spermatozoa in histological preparations of *H. grisea* were presented by Newton (1970). Spermatozoa of other plagiostomids have been figured or described by several authors in taxonomic studies (Bresslau, 1933; Hyman, 1938; Kepner, Stirewalt and Ferguson, 1941; Stirewalt, Ferguson and Kepner, 1942; Westblad, 1956; Karling, 1962; Kulinich, 1970, 1973). Plagiostomid spermatozoa are included in phylogenetic schema which have resulted from efforts at comparative spermatology (Hendelberg, 1969; 1970; *cf.* Bedini and Papi, 1970). Christensen (1961) provided the only other account of the ultrastructure of the spermatozoön of a plagiostomid turbellarian: the marine form *Plagiostomum morgani,* from Woods Hole, Massachusetts. Unfortunately, Christensen's brief abstract was not accompanied by electron micrographs.

Living mature spermatozoa of some marine plagiostomid species have been described by Jensen (1883), Böhmig (1891) and von Graff (1908, 1911). Only one of these authors, Jensen, observed movement by a spermatozoön.

Absence of flagella in the spermatozoa of plagiostomid turbellarians (Christensen, 1961; Hendelberg, 1969) requires another structural basis for sperm motility. The presence of "fibrils or tubules" immediately beneath the plasma membrane of the spermatozoön of *P. morgani* led Christensen (1961) to suggest that the region containing the tubules (*i.e.*, "the pellicle") is the basis for sperm motility in *Plagiostomum.* Cortical singlet microtubules in the aflagellate spermatozoön of *Hydrolimax grisea* are reported in this communication. Additional ultrastructural details, of sperm nucleus and of cytoplasmic specializations, suggest a unique morphology for the unusual male gamete of *Hydrolimax.*

MATERIALS AND METHODS

For bright-field microscopy, specimens of *Hydrolimax* were prepared according to the procedures of Newton (1970). Wet mount preparations of live

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[1] This paper is dedicated to Dr. Donald P. Costello on the occasion of his retirement and in celebration of his forty years of teaching at the University of North Carolina.

[2] Research performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology, University of North Carolina at Chapel Hill, under the direction of Drs. Donald P. Costello and Catherine Henley.

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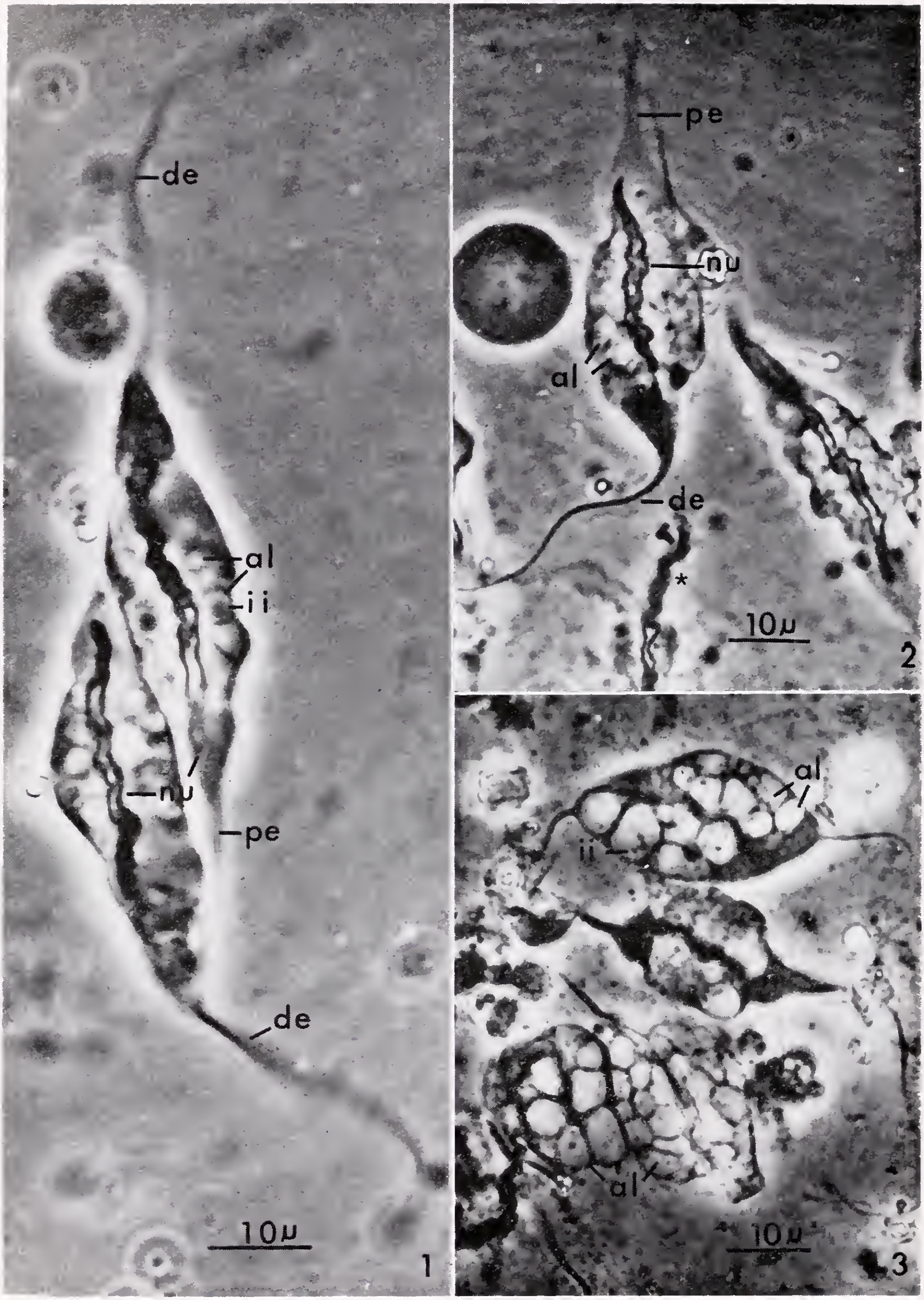
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FIGURES 1–3. Phase contrast micrographs of living mature spermatozoa of *Hydrolimax grisea* released into pond water.

spermatozoa were studied and photographed with a Zeiss Photomicroscope I, using phase optics.

Two methods of fixation for electron microscopy were used: the procedure of Anderson and Personne (1970) for freshwater material; and a procedure of D. P. Costello (personal communication; described in Newton, 1974). The material was dehydrated in a graded series of ethanol, passed through propylene oxide and embedded in Epon-Araldite. Sections were cut on a Sorvall Porter-Blum MT-2 ultramicrotome with glass or diamond knives and stained with 3% uranyl acetate alone or in combination with 0.5% lead citrate. Maceration and negative staining of spermatozoa were done in 1% aqueous solution of phosphotungstic acid according to the method of Henley (1970). Both sectioned and negatively stained preparations were studied with a Zeiss 9S2 or an Hitachi HU-11B electron microscope.

OBSERVATIONS AND RESULTS

Light microscopy

The fixed, sectioned and stained male gamete of *Hydrolimax* has a central core of Feulgen-positive chromatin. This nucleus is fuchsinophilic and the cytoplasm stains light blue after Mallory's triple stain. In Giemsa smear preparations and with iron hematoxylin sperm cytoplasm is unstained but marked by a distinct boundary. All these observations were reported previously (Newton, 1970). One end of the nucleus is rounded in contour, whereas the other end terminates in a sharp point.

When observed with phase optics, however, the living mature spermatozoön of *H. grisea* is considerably more complex in structure (Figs. 1–3) than is shown in the usual histological preparations. The size of the spermatozoön varies from 80 to 100 μ in length and from 9 to 13 μ in width. The cell body is fusiform but irregular in contour. The vermiform nucleus extends almost the full length of the gamete and is surrounded by alveolate cytoplasm. Within the alveoli of the sperm cytoplasm are spherical or irregular inclusions (Figs. 1 and 3) which, in living spermatozoa, exhibit Brownian movement.

No flagella were seen in the spermatozoön of *Hydrolimax*. Movement by the spermatozoön was not observed. What is "head" and "tail" of the gamete remains unknown. For purposes of description, the ends of the spermatozoön are here designated proximal and distal with respect to a residual body to which developing spermatozoa are attached (*cf.* Jensen, 1883; Böhmig, 1891). The proximal end of the spermatozoön is circular in optical transsection and is translucent. The

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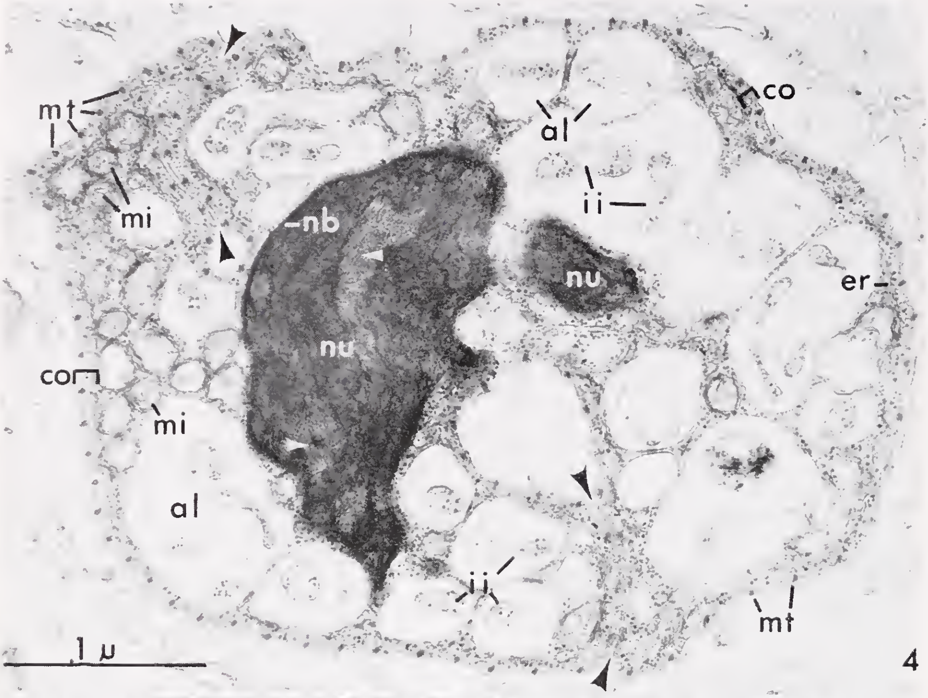
FIGURE 1. Spermatozoa with spherical inclusions in alveoli. Distal ends extend out of plane of focus. The following abbreviations represent: al, alveolus; de, distal end; ii, intra-alveolar inclusion; nu, sperm nucleus; and pe, proximal end.

FIGURE 2. Distal end of spermatozoön is in "side" view. Compare with proximal end. Asterisk marks a nucleus of a cytolyzed spermatozoön; note attached debris. Abbreviations are as in Figure 1.

FIGURE 3. Alveoli, and irregular inclusions, become more distinct when sperm are compressed by weight of overlying coverslip. One spermatozoön (center) has cytolyzed. Abbreviations are as in Figure 1.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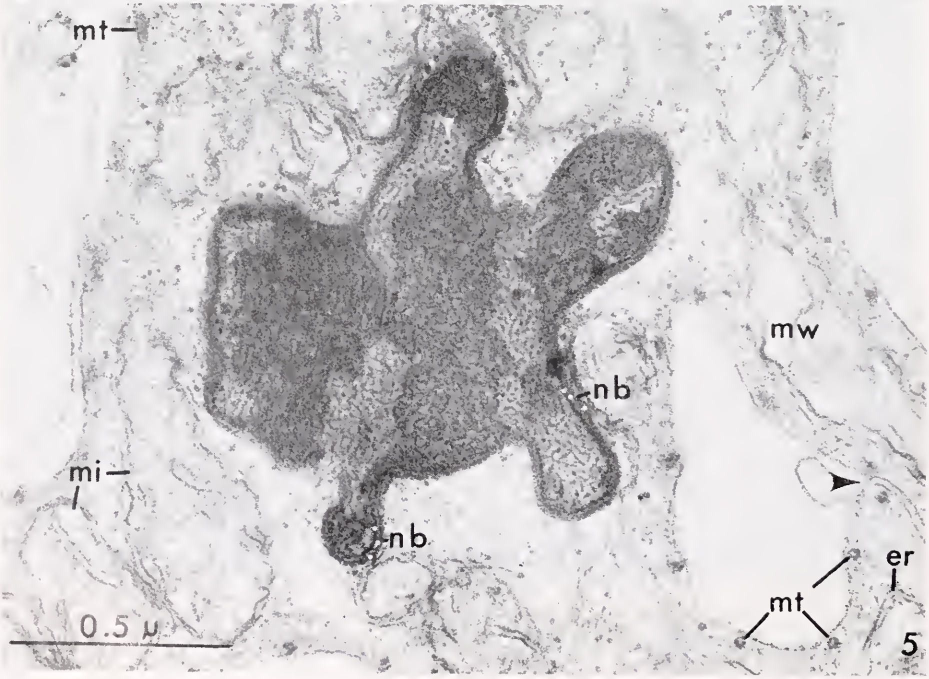
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elongate distal end is flattened, as can be verified by careful examination of the spermatozoön through many planes of focus, and is often bent in the plane perpendicular to the plane of flattening.

Electron microscopy

In the parenchyma of adult turbellarians which have recently copulated, spermatozoa aggregate in the vicinity of oöcytes outside the oviducts (Newton, 1970; Fig. 17; *cf.* Christensen, 1961). From sections of such aggregations, spermatozoan ultrastructure can best be studied, for these male gametes are undoubtedly mature: the spermatozoa have migrated through the parenchyma to fertilize the eggs.

In cross-section, the spermatozoön of *Hydrolimax* has an irregular boundary (Fig. 4), the outline of which varies among spermatozoa and among different sections of the same spermatozoön. Three major ultrastructural features may be distinguished in sections of the gamete: (1) the cortex, with its many microtubules; (2) the alveolate cytoplasm; and (3) the nucleus.

The cortex of the spermatozoön of *Hydrolimax* includes cytoplasm immediately beneath the cell surface containing the microtubules (Figs. 4 and 6). The inner boundary of the cortex is usually marked by a double membranous structure, apparently smooth endoplasmic reticulum. Other organelles, including mitochondria and alveoli, are found beneath the cortical cytoplasm. The cortex is of medium electron density, granular and varies from 430 Å to over 1000 Å in thickness. An indication of special physical properties of cortical cytoplasm is the absence of organelles, other than microtubules, from the cortex. Often the cortex is folded in toward the nucleus (Figs. 4 and 5), especially in the proximal half of the cell. In longitudinal sections of spermatozoa the cortex is often in close proximity to the nucleus for relatively long distances. Infolding of the sperm cortex may serve to divide the cytoplasm of the spermatozoön into lateral cytoplasmic extensions (*cf.* Hyman, 1938). In the spermatozoan mid-region, the cortex does not fold in toward the nucleus.

Figures 7 and 8 are micrographs of successively distal sections of spermatozoa. The distal terminus of the sperm nucleus is seen in section in Figure 7, along with some membranous elements, here difficult to identify. The cortex is thrown into several folds toward the nucleus. A transverse section through the flattened distal end of the spermatozoön (Fig. 8) shows an endoplasm devoid of organelles, except for a suggestion of endoplasmic reticulum.

Within the cortex singlet microtubules spiral along the length of the spermatozoön (Figs. 4, 5, and 6). In any transverse section of a spermatozoön, the cortical singlet microtubules exhibit varying orientations with respect to the plane

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FIGURE 4. Electron micrograph of transverse section of proximal end of spermatozoön. The cortex folds in toward the nucleus in two places (large arrows). Small white arrows point out fibrous component of nucleus. Approximately 98 microtubular profiles can be counted in the cortex. Costello's fixative is used; uranyl acetate is used; co represents cortex; er, endoplasmic reticulum; mi, mitochondria; mt, microtubule; nb, nuclear boundary; other abbreviations as in Figures 1–3.

FIGURE 5. Transverse section of sperm nucleus (*cf.* Fig. 4) showing dark fibers (small arrows) in cross section. Large arrow points to infolding of cortex. Costello's fixative is used; uranyl acetate is used; mw represents membranous whorl; other abbreviations as in Figures 1–4.

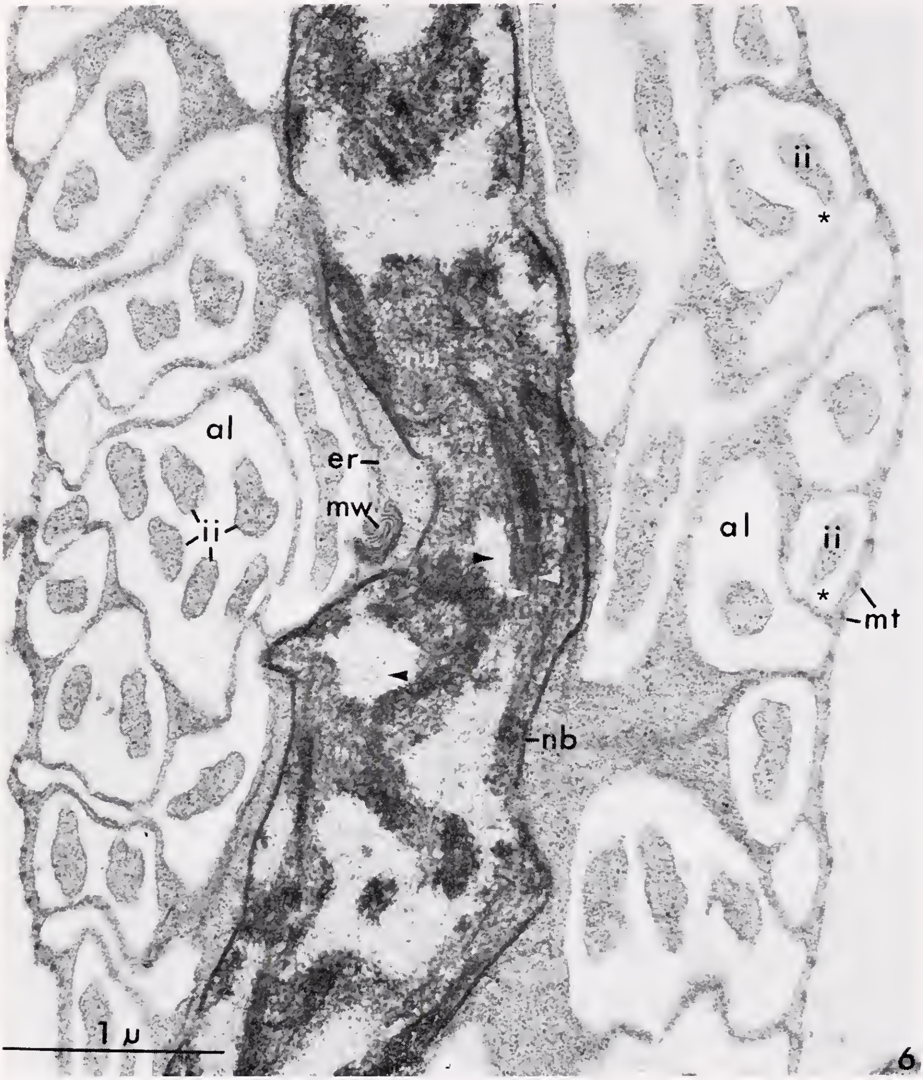
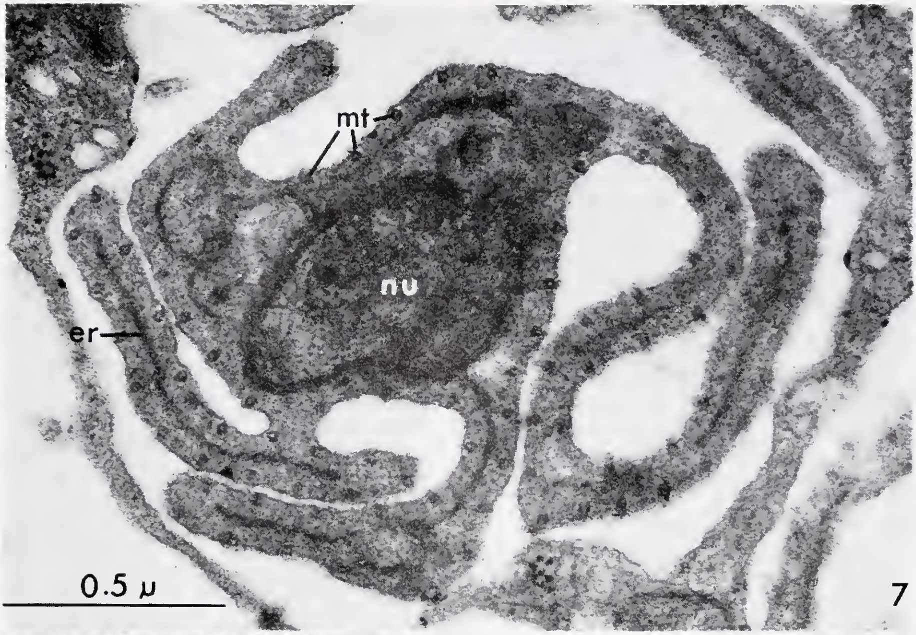


FIGURE 6. Longitudinal section of a spermatid mid-region. Arrows point to dark fibers of nucleus. Asterisks indicate connections between intra-alveolar particles and sperm cytoplasm. Anderson-Personne fixative is used; uranyl acetate, and lead citrate are used; abbreviations are as in Figures 1–5.

of section, from perpendicular to oblique, to parallel (Figs. 4 and 5). The longitudinal section in Figure 6 shows microtubules in transverse section. The number of cortical singlet microtubules in cross-sections of spermatozoan mid-regions ranges from 90 to 116. The singlets are widely separated in the sperm mid-region, *ca.* 1200 to 1800 Å, and closely spaced in the distal end, *ca.* 100 to 200 Å (*cf.* Figs. 4 and 8). There is no evidence of bridging between microtubules. No connection between



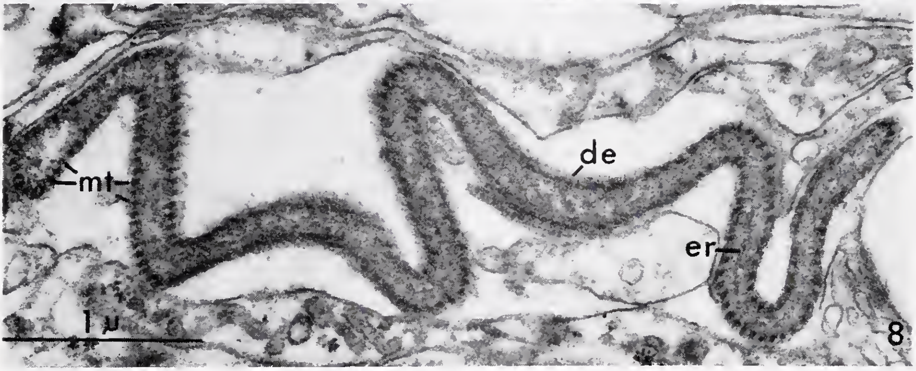


FIGURE 7. Distal nuclear terminus is surrounded by sparse cytoplasm with some membranous elements. Cortex is highly folded. Costello's fixative is used; uranyl acetate is used; abbreviations are as in Figures 1–5.

FIGURE 8. Transverse section of flattened distal end. Note numerous microtubules. Costello's fixative is used; uranyl acetate is used; abbreviations are as in Figures 1–5.

the singlets and the sperm cell surface is seen. An accurate count of microtubules is difficult due to vagaries in the shape of the spermatozoön and to oblique orientation of microtubules in the plane of section. The outside diameter of microtubules, calculated from measurements from transverse sections, is *ca.* 275 to 320 Å.

Maceration and negative staining of spermatozoa (from dissected seminal vesicles) provided excellent material for examination of cortical microtubules in the distal region of the spermatozoön (Figs. 9–12). Remnants of the sperma-

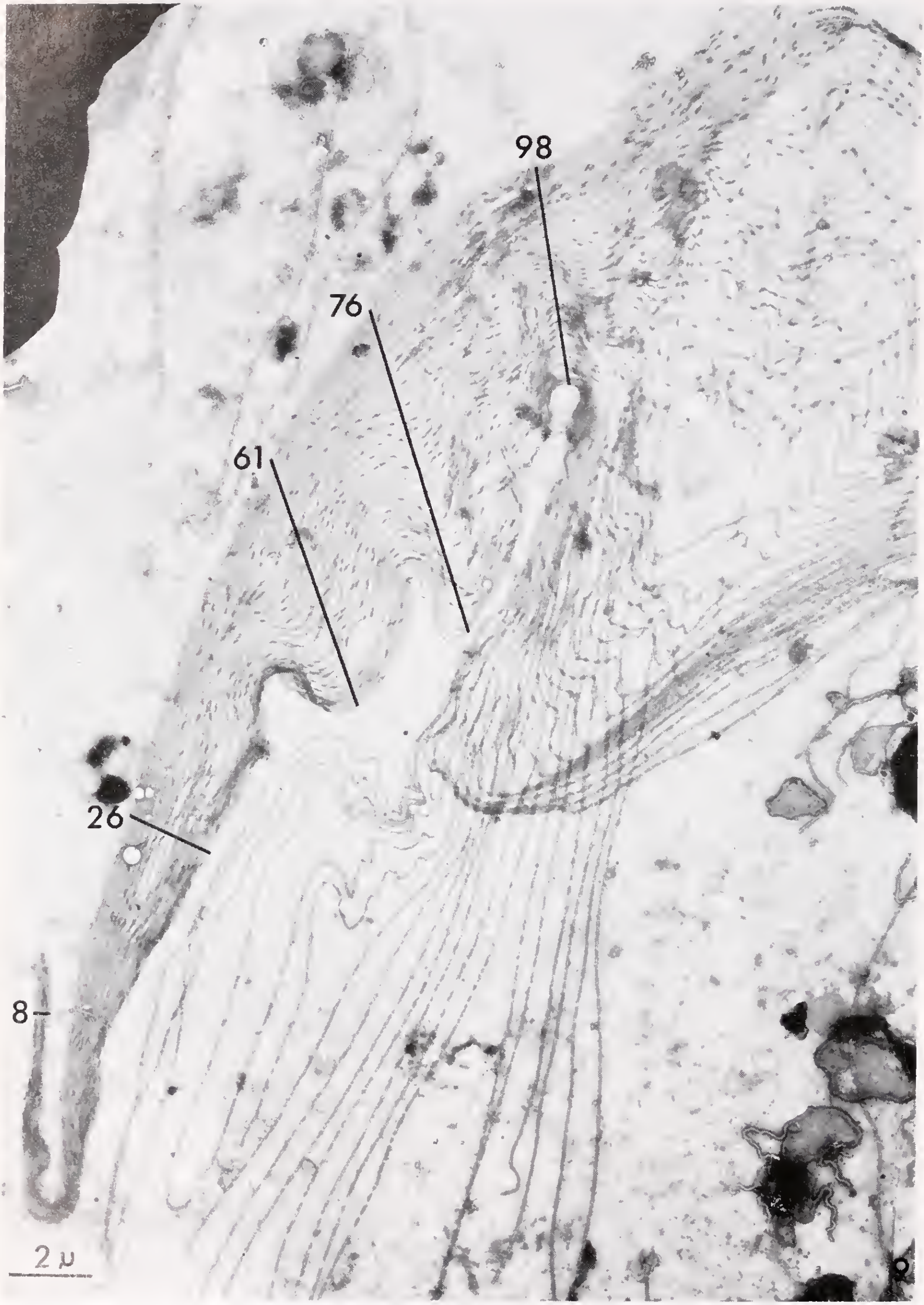


FIGURE 9. Cortical singlet microtubules revealed by maceration and negative staining with PTA. Numbers refer to microtubules in transects. Some of the microtubules are relatively straight whereas others noticeably bend into a wave pattern.

tozoan nucleus and the alveolate cytoplasm of the mid-region were extremely electron-dense after treatment with PTA. A few well macerated and spread distal ends of spermatozoa permitted a count of up to 114 microtubules. The microtubules terminate abruptly and decrease in number rapidly toward the end of the spermatozoön (Fig. 9).

Figure 10 demonstrates different orientations of microtubules within different regions of the same sperm cell. Apparently the distal end of the spermatozoöon was reflected back alongside the mid-region during maceration with PTA. The cortical singlets within the distal end are straight, at least more so than within the spermatozoan mid-region. It is interesting that a microtubule is straight in the distal end and spirally arrayed in the cortex of the mid-region of the spermatozoön. PTA digests of the proximal end likewise show straight microtubular arrays. The cortical singlet microtubules "change" their orientation from spiral to straight as they extend proximally or distally from the mid-region.

The spermatozoan microtubules in *Hydrolyimax* are *not* rigid structures: the cortical singlets bend. In Figure 10, some microtubules of the distal end are straight, whereas others have an irregular wave-like pattern. A similar microtubular arrangement of straight and "wavy" cortical singlets can be seen at lower magnification in Figure 9. In Figure 11 the radii of the microtubular arc shown at the arrows are approximately 0.46 μ—the smallest observed arc radius into which microtubules were bent. Bending through an arc radius of less than *ca.* 0.46 μ may produce breaks, likewise evident in Figure 11. Microtubule bending may have been caused by action of PTA or created during removal of macerating fluid.

The diameter of singlet microtubules in PTA preparations is *ca.* 270 to 300 Å The cortical singlets have a substructure suggested by alternately light and dark bands pitched relative to the microtubule axis. The electron-lucent bands, bounded by electron-opaque deposits of phosphotungstate, are the only subunits observed in the present material (Fig. 12; *cf.* Thomas and Henley, 1971). These subunits have a center-to-center spacing of 79 to 86 Å and pitch angles of 10° to 22°. No protofilaments were observed (see, however, Fig. 12). A dark line, *ca.* 34 to 40 Å wide, extends down the center of the singlets (Figs. 11 and 12). This striking linear feature of the microtubules may represent the "lumen" filled with electron-opaque phosphotungstate. It is possible that some substance within the singlet has a special affinity for PTA. The dark line appears to divide the subunits, creating the illusion of a "herring-bone" or double-element array of substructure. The subunits are not divided, however, but apparently are spirally arrayed round the central element of the microtubule, the "lumen."

The cortical singlets are apparently quite long; lengths up to 70 μ can be estimated from Figure 9 (the estimates include *only* the portions of the microtubules in the figure). Examination of a series of micrographs in a montage suggests microtubule lengths up to 100 μ or more. Single microtubules can extend from one end of the spermatozoön to the other.

The cytoplasmic alveoli of the spermatozoön are membrane-bound vesicles (Figs. 4, 5 and 6), in which are suspended membrane-bound inclusions of coarsely granular material. The alveoli also contain irregularly shaped particles of medium electron density. The significance of the alveoli and of their contents is unknown. Current studies of spermiogenesis at the EM level indicate that the alveoli originate

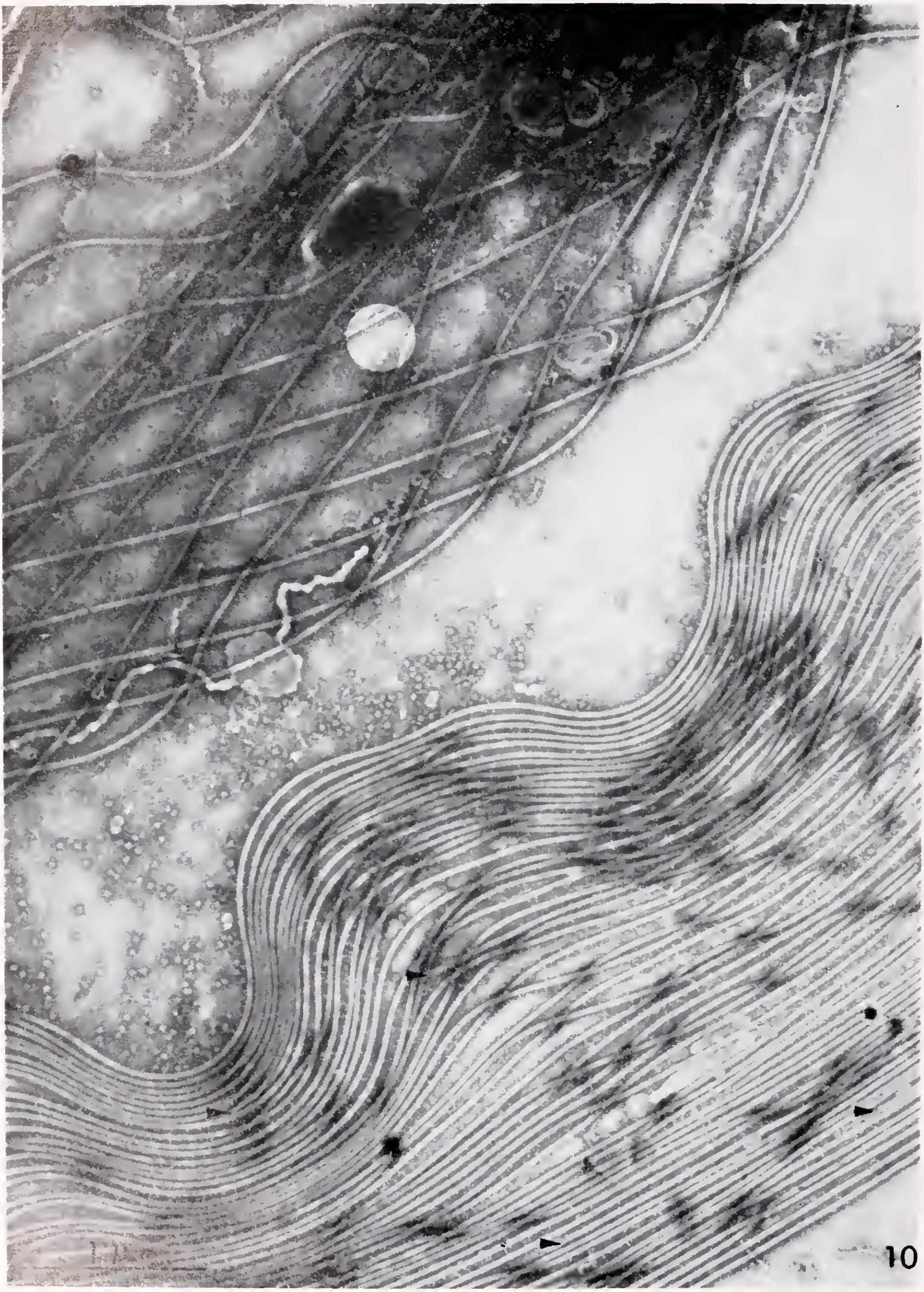


FIGURE 10. Cortical singlet microtubules of the mid-region of the spermatozoön have settled on the Formvar-carbon-coated grid in an apparent geometric projection of their spiral course along the sperm-cell body (top). In contrast, the microtubules of the distal end (bottom) do not spiral along their lengths but show "straight" or "wavy" configurations. Arrows point to microtubule termini.

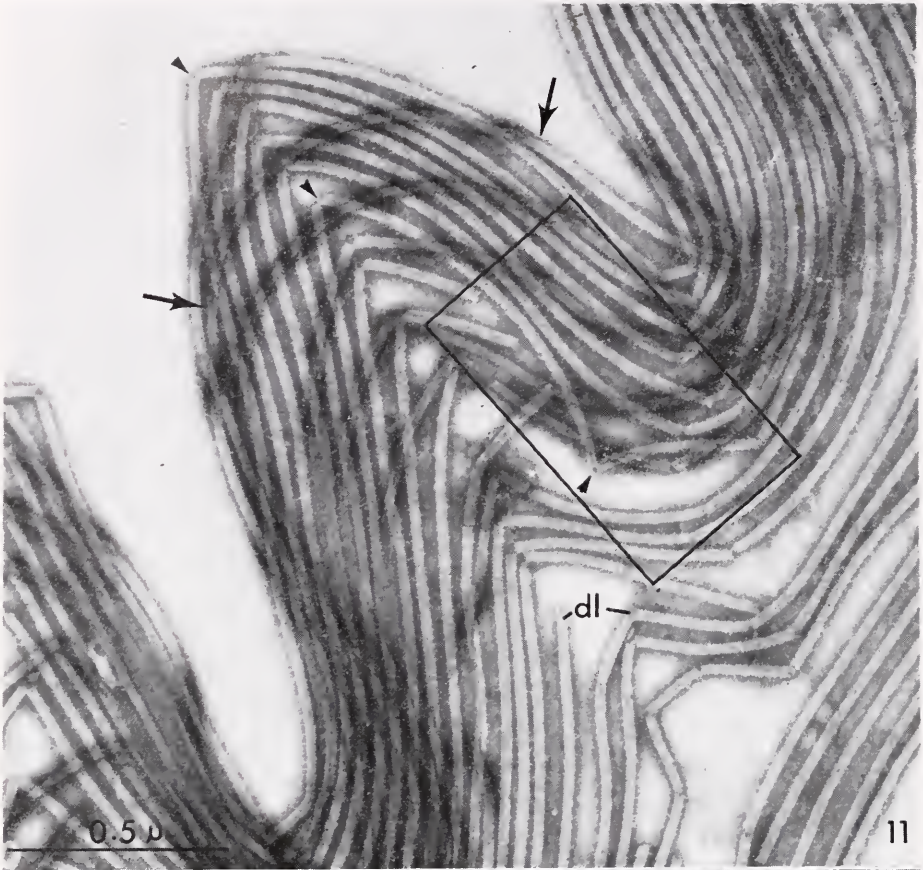
as invaginations from the surface of the spermatid and that secondary cytoplasmic extensions into the alveoli give rise to intra-alveolar cytoplasmic particles (Fig. 6). Mitochondria are included among the alveoli in the sparse cytoplasm of the spermatozoan mid-region. The alveoli do not extend into the ends of the seprmatozöon. In the proximal region of the gamete, membranous whorls, endoplasmic reticulum and many mitochondria are found within coarsely granular cytoplasm (Fig. 5).

In overall appearance the nucleus is electron-dense and therefore stands out in marked contrast to the cortical and alveolar regions of the spermatozoön (Figs. 4, 5 and 6). The nucleus is irregular in cross-sectional outline and is bounded by an envelope approximately 260 Å in thickness (Figs. 4 and 5). The nuclear contents are not uniformly electron-dense. In any section of the nucleus, electron-lucent and electron-opaque areas are observed in an apparently random pattern (Figs. 3, 4 and 5). Within electron-lucent areas, in transverse sections especially, fibers are seen (Fig. 4); these fibers are predominantly longitudinal in orientation (*cf.* Fig. 6). A similar fibrous component can be discerned within electron-opaque regions of the sperm nucleus. The fibers vary in diameter from 80–120 Å and are separated from one another by 110 to 130 Å (average 123 Å). A granular dark material provides the background for electron-opaque regions. The nucleus of a spermatid (Fig. 6) is not as electron-opaque as the nucleus of mature sperm (*cf.* Figs. 4 and 5). A longitudinal array of dark fibers is evident in dark and light areas of the condensing sperm nucleus. A few fibers are cut transversely in Figure 6. What nuclear components (as here defined by morphologic and electron-density criteria) constitute sperm chromatin in this form is not clear.

DISCUSSION

In the classical sense, the spermatozoön of *Hydrolimax* cannot be said to have a head, neck, middle piece or tail (*cf.* Wilson, 1925). No structure resembling or functioning as an acrosome has been observed, nor have flagella or flagella-like bodies been observed in the mature spermatozoön or at any time/stage of spermatogenesis (Newton, unpublished data). Spermatozoa removed from the body of *Hydrolimax* into pond water exhibit no motility.

Jensen (1883), Böhmig (1891) and von Graff (1908, 1911) described living mature spermatozoa of some marine plagiostomid species. Except for spiraling of the sperm-cell body, reported by Jensen (1883) for *Plagiostomum vittatum* and by Böhmig (1891) for *P. girardi* and *P. reticulatum,* the spermatozoa of the marine species are morphologically similar to the spermatozoön of *Hydrolimax*. Böhmig (1891) observed that the central core (*Centralfaden* = sperm nucleus) of plagiostomid spermatozoa in histological preparations is composed of a proximal darkly-staining region and a distal lightly-staining region after hematoxylin and carmine stains. The proximal and distal regions were, according to Böhmig, composed of chromatic and achromatic nuclear substances. Jensen (1883) did not report regional variation in staining properties in the sperm nucleus of *P. vittatum;* none has been observed in spermatozoa of *Hydrolimax* in histological preparations. Böhmig (1891) and von Graff (1908, 1911) describe and figure spiral nuclei in spermatozoa in histological preparations of some marine plagiostomids. Spiral nuclei were not reported by Jensen (1883), and none was observed in the present study.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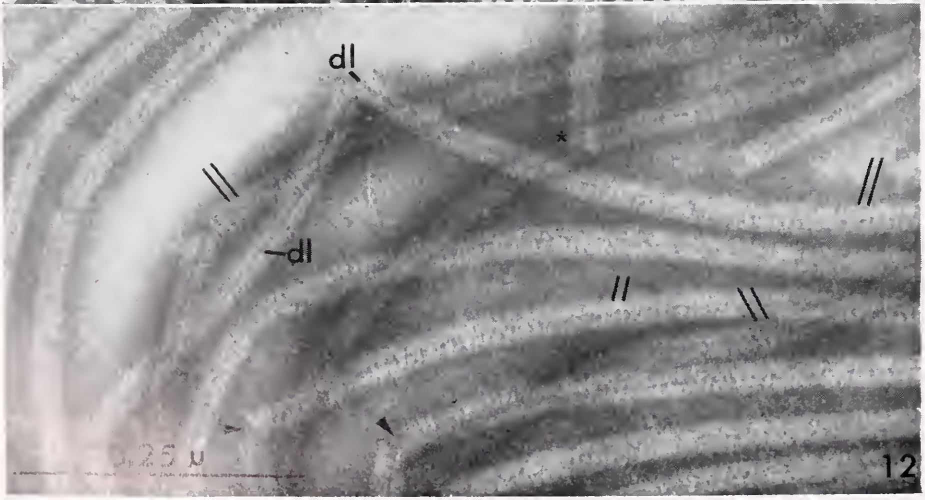
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Christensen (1961; p. 416) briefly described the mature spermatozoön of *Plagiostomum morgani:* in general morphology the spermatozoön of *P. morgani* is like that of *H. grisea,* ". . . spindle-shaped, tapering at each end into slender processes which are respectively 40 μ and 10 μ." The short and long processes may correspond to the proximal and distal ends, respectively, of the spermatozoön of *Hydrolimax.* The nucleus of the spermatozoön of *P. morgani* is fusiform and contains a "dense core" (electron-dense?) which protrudes from the end of the nucleus at the shorter end of the spermatozoön. No such dense nuclear core, nor protrusion, is seen in the nucleus of the spermatozoön of *H. grisea.*

After extensive molecular reorganization and condensation, the nuclear contents of the spermatozoa of most animals becomes uniformly electron-dense (André, 1963; Walker and Macgregor, 1968; Chevallier, 1970; Fawcett, Anderson and Phillips, 1971; Henley, 1973). There are exceptions, however, of which the nuclear contents of the spermatozoöon of *Hydrolimax* is another example. Von Bonsdorff and Telkka (1965; their Fig. 3) show the sperm nucleus of *Diphyllobothrium latum* to be electron-lucent, with scattered, finely-fibrillar, electron-dense material. Henley (1974) described the sperm nucleus of the polyclads *Stylochus* and *Notoplana* as diffuse, showing a lamellar array of electron-opaque fibers in an electron-lucent background (*cf.* Henley, 1974; Fig. 19). The nuclei of the spermatozoa of the acoels *Anaperus* and *Polychoerus* are "diffuse," without lamellar arrays of fibers. In *Polychoerus,* the spermatozoa have an electron-dense nuclear periphery (Henley, 1974; Fig. 21). Silveira and Porter (1964) were able to resolve the nuclear contents of the spermatozoa of *Dugesia tigrina, Bdelloura candida* and *B. propinqua* into two components: (1) a dense component constructed of lamellar or leaf-like subunits 60 Å thick, and (2) a less dense, finer-textured component. On the basis of staining affinity for uranyl acetate, Silveira and Porter (1964) concluded the dense component to be "chromatinic" and the lucent component to represent residual protein. In transverse section ". . . the chromatinic material comprises frequently a 'cruciform' figure, with four less dense areas representing the protein components disposed peripherally in the four quadrants of the cross . . ." (Silveira and Porter, 1964; p. 246). In a subsequent study of the nucleus of the spermatozoön of *D. tigrina,* Silveira (1970) characterized the proteinic (electron-lucent) nuclear component as a basic protein which she tentatively interpreted as being a homologue of an acrosome.

The "pellicle" (= cortex; *cf.* Christensen, 1961; p. 416) of the spermatozoön of *P. morgani* contains ". . . parallel fibrils or tubules . . . which pursue a spiral course up the body of the sperm." The cytoplasm of the mid-region of the spermatozoön of *P. morgani* ". . . contains whorls of flattened cisternae, resembling endoplasmic reticulum (without ribosomes), which extend out to insert along every other or every few pellicular fibrils" (Christensen, 1961; p. 416). Membranous whorls are observed occasionally in the proximal portion of the spermatozoön of *H. grisea,* but they do not appear to insert along the cortical singlet microtubules.

FIGURE 11. Singlet microtubules showing curves (large arrows) and breaks (small arrows). A dark line, probably PTA deposits, extends down the singlet centers. Outlined area is shown at higher magnification in Figure 12; dl represents dark line.

FIGURE 12. Pitch and spacing of subunits (parallel lines) suggest a helical substructure to singlets. A linear (protofilamentous) array is indicated at asterisk. Arrows indicate breaks; dl represents dark line.

Alveoli characterize the cytoplasm of the mid-region of the spermatozoön of *Hydrolimax*. On the presence or absence of centrioles in the spermatozoön of *P. morgani*, Christensen (1961) makes no comment. No centriole has been observed in any section of spermatozoa of *H. grisca* studied by electron microscopy. Likewise, centrioles have not been found in spermatozoa studied by light microscopy, in squashes, smears or sectioned and stained material.

Christensen (1961) found no evidence of flagella or of an undulating membrane in the spermatozoön of *P. morgani*. Hendelberg (1969) did not find flagella in the spermatozoa of other plagiostomids, *P. vittatum*, *P. cinctum*, *Vorticeros auriculatum* and *Acomostomum dioicum*, studied by phase microscopy.

Flagella and axonemes are absent from spermatozoa of a number of other turbellarian forms. The spermatozoa of *Macrostomum rubrocinctum*, studied by phase optics only, are aflagellate (Hendelberg, 1969). In a freshwater species of *Macrostomum*, collected near Chapel Hill, North Carolina, the spermatozoa, studied by phase-contrast and electron microscopy, are non-axonemal (Thomas and Henley, 1971). In some rhabdocoels, *Provortex balticus*, *Graffila buccinicola*, *Syndesmis echinorum* and *Proxenetes* sp., the spermatozoa are aflagellate as Hendelberg (1969) concluded from phase-contrast microscopy. Spermatozoa with no free flagella may have axonemes incorporated into the cytoplasm along the length of the body.

Mesostoma georgianum (Henley, Costello, Thomas and Newton, 1969), *Dugesia tigrina*, *Bdelloura candida* and *B. propinqua* (Silveira and Porter, 1964), and *Microdalyellia* sp. (Henley, 1974) have biflagellated spermatozoa, the bodies of which undulate independently of the two flagella. Spermatozoa of *Stylochus zebra* have no free flagella; they do have lateral cytoplasmic axonemes (Thomas, 1970). Cortical singlet microtubules are longitudinally aligned along the bodies of all these spermatozoa. Hendelberg (1970) observed, however, that the undulations of the body of the biflagellated spermatozoa of some polyclads cease when flagella are removed by mechanical means.

Mattei (1970) and Mattei, Mattei, Reizer and Chevalier (1972) reported aflagellate spermatozoa from six species of teleosts from Africa. In only one of the species, *Gymnarchus niloticus*, were cortical singlet microtubules observed in the spermatozoa; and in *G. niloticus* the spermatozoa exhibited a kind of ameboid motion. Mattei *et al.* (1972) were not able to observe living spermatozoa of the other teleosts.

Axonemes of the common "9 + 2" pattern of microtubules are conspicuously absent from the spermatozoa of coccid insects (Moses, 1970; Robison, 1972). Longitudinally-oriented singlet microtubules, concentric or spiral in transverse array, are the motile element of coccid spermatozoa (Moses, 1970; Robison, 1972). In the aflagellate spermatozoön of the dipteran, *Rhynchosciara* sp., a "flagellar complex" of over 350 doublet microtubules constitutes the motile apparatus (Shay, 1972).

The spermatozoa of *Macrostomum* sp. (Thomas and Henley, 1971), of *M. retortum* and *Paramacrostomum quiesztori* (Bedini and Papi, 1970), with cortical singlets as their only microtubular elements, are the only verified non-axonemal sperm of Turbellaria known to be motile. There exists a tacit implication of motility in the spermatozoa of the other turbellarians discussed above. Hendelberg

(1970) notes that the aflagellate spermatozoa of some Turbellaria are capable of active motility, especially the spermatozoa of the plagiostomids. There is only one description in the literature, of which the present author is aware, of movement by the aflagellate spermatozoön of a plagiostomid; Jensen (1883; p. 22) made one observation of "legers mouvements d'oscillations de la queue" of a spermatozoön of *Plagiostomum vittatum*. In the absence of more direct observations, it is assumed that, since the sperm are injected into the parenchyma of copulating adults, the spermatozoa actively migrate to the vicinity of eggs. The spermatozoa may require some sort of capacitation or other activation within the maternal parenchyma to become motile. The importance of the substrate through which plagiostomid spermatozoa travel under normal conditions to effect fertilization cannot be overlooked. The interaction between spermatozoa and parenchymal cells of adult turbellarians remains to be investigated. Christensen (1961, p. 416) assumed the spermatozoa of *P. morgani* to ". . . proceed by some sort of wriggling motion." He proposed the cortex to be ". . . the basis of motility, perhaps through differential contractions of the pellicular fibrils over the surface of the sperm" (Christensen, 1961; p. 416). Possibly the cortical singlets, alone or in concert with some cortical factor or substance, are responsible for motility in aflagellate, non-axonemal turbellarian spermatozoa.

How the sperm move is unknown. If cortical singlet microtubules, reported in spermatozoa of *Plagiostomum* and *Hydrolimax*, are responsible for movement, then the question is: how? In the mid-region of the sperm of *Hydrolimax*, the microtubules are widely separated. In the compressed distal portion of the sperm microtubules are close. No evidence of bridging between microtubules has been observed in *Hydrolimax*. The "fibrils or tubules" of the spermatozoön of *Plagiostomum* are spaced 700 Å apart (Christensen, 1961). The distances over which microtubules are capable of interacting are unknown. There is a possibility that singlet microtubules reported here may serve *solely* as cytoskeletal elements.

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SUMMARY

1. The structure of the aflagellate, non-axonemal spermatozoön of *H. grisea* was studied by phase contrast optics and electron microscopy.

2. The gamete is fusiform with a centrally-located, vermiform nucleus surrounded by cytoplasmic alveoli.

3. The alveoli and intra-alveolar inclusions are membrane-bound vesicles and membrane-bound particles, respectively. The latter resemble sperm cytoplasm and are believed to be cytoplasmic extensions into alveoli.

4. The nucleus has an irregular pattern of electron-opaque and electron-lucent areas. In predominantly longitudinal orientation are dark fibers, *ca.* 95 Å separated by 123 Å, coursing through the opaque and lucent areas of the nucleus.

5. Cortical singlet microtubules are the only microtubular elements in the spermatozoön of *Hydrolimax*. The array and discernible substructure of the singlets are described from sectioned and PTA-macerated material. The *possible* role of the singlet microtubules in sperm motility in *H. grisea* is discussed.

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DYNAMICS OF JUVENILE HORMONE ACTION IN LARVAE OF THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.)

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In insects the maintenance of larval characters depends on the presence of active *corpora allata* (CA) (Williams, 1961; Wigglesworth, 1970). The implication is that adequate titers of juvenile hormone (JH) in the hemolymph and tissues are necessary for the maintenance of the larval state. The *status quo* action of JH (Williams, 1963a) has, until recently, been the only well established role for this hormone during larval life. We could regard this role of JH as a "passive" one since its action becomes evident only when an ecdysone-induced molt occurs. In apparent contrast stands the positive role of JH in final-instar larvae of certain Lepidoptera where JH inhibits the secretion of the prothoracicotropic hormone (PTTH) by the brain (Nijhout and Williams, 1974b). It is of interest, therefore, to consider in further detail the requirement for JH in these two processes. The approach taken in the present study was the premature elimination of the source of JH in final instar larvae. The expectation was that such action would lead to an early release of PTTH and would also allow ecdysone to induce prematurely the switchover from larval to pupal development (Truman, Riddiford and Safranek, 1974). The surprising finding was that the mere absence of JH was not a sufficient condition for either of the above-mentioned events. An unidentified time-dependent process must apparently be completed to eliminate the effects of JH on the target tissues.

MATERIALS AND METHODS

Rearing procedure

Larvae of *Manduca sexta* were reared as described by Truman (1972). All experimental animals were kept at 25° C under a short-day (12L:12D) photoperiod regimen.

JH extraction

Hemolymph. Isolated abdomens were prepared by ligating CO_2-anesthetized larvae between the 2nd and 3rd abdominal segments with waxed dental floss. The part of the larva anterior to the ligature was always removed. Ligations were performed 4–5 hours after lights-on. Hemolymph from unligated larvae was taken at the same time. For JH extraction the hemolymph of 12–16 larvae was pooled and 7 ml of this sample was extracted and purified according to the procedure of Fain (1975). The final lipid residue was dissolved in 28 μl of cyclohexane and stored at −20° C. All extracts were assayed within five days. By suitable tests it was possible to show that no measurable activity was lost during this period.

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Blood-free carcass. Anesthetized larvae were slit open, the gut and adhering Malpighian tubules were removed, and the carcass was blotted with filter paper. Fat body, muscle and epidermis were removed from 10–14 such carcasses by means of a roller technique devised by Dr. J. S. Bjerke (Harvard University, personal communication). The pooled tissues were disrupted by sonication at 90 W for a total of 60 sec. Seven ml of the brei were extracted according to the procedure of Fain (1975). Forty μl of the lipid residue were partially purified on glass-backed, silica gel, thin layer chromatography plates (EM laboratories). Chromatograms were developed four times in the same direction with 20% ether-hexane. The portion of the chromatogram corresponding to the position of a tritiated C18-JH (New England Nuclear, 11 C per m mole) marker was eluted with distilled ethyl acetate. After evaporation of the ethyl acetate the lipid residue was dissolved in hexane and passed over a column of activity IV alumina (Woelm). The hexane was then evaporated off and the residue dissolved in an equal volume of cyclohexane and stored at −20° C.

JH assay

The JH extract was assayed on larvae of a black strain of *Manduca* isolated from a mutant which arose spontaneously in our stock. This mutant is expressed as a deficiency in the secretion of JH by the larval CA during the molt to the 5th (final) larval instar (Safranek and Riddiford, 1975). Sufficient JH is present at the time of apolysis to ensure a larval molt, but 17–25 hours later, when the JH titer in the blood of wild type larvae declines to a low level it falls to undetectable levels in the mutant (Safranek and Riddiford, 1975) so that no JH is present during the critical period for normal pigmentation (Truman, Riddiford and Safranek, 1973). The black phenotype can be completely reversed by application of JH during this critical period resulting in larvae of green (wild type) coloration (Safranek and Riddiford, 1975). The response of the black mutant to exogenous JH is identical in all respects to that of larvae used in the black larval assay of Truman *et al.* (1973). Further description of the black mutant is given by Safranek and Riddiford (1975). The bioassay and scoring system are described in detail by Fain and Riddiford (1975).

One microliter of the hemolymph extract or 3 μl of the blood-free carcass extract was applied to each of 20–25 test larvae. Their response was scored three days later. The mean score and 95% confidence limits were calculated for the combined data of three replicates of each experiment. Assay of a dilution series of a zero hour sample produced a dose-response curve that paralleled the one for C18-JH. All data were converted to C18-JH equivalents on the basis of a dose-response curve determined by Fain (1975). Although C18-JH has not been detected in *Manduca* (Judy, Schooley, Dunham, Hall, Bergot and Siddall, 1973), the activity of this hormone has been found to be identical to that of C17-JH in the various bioassays developed on *Manduca* (Riddiford and Ajami, 1973; Nijhout and Riddiford, 1974). A proper dose-response curve for C17-JH could not be prepared due to the unavailability of enough of this hormone. The CA of *Manduca* adult females *in vitro* produce C16-JH and C17-JH in roughly equal proportions (Judy *et al.,* 1973), but C17-JH is 200–300 times more active on *Manduca* than C16-JH (Riddiford and Ajami, 1973; Nijhout and Riddiford, 1974; Fain, 1975). It is therefore likely that C17-JH accounts for virtually all of the JH activity in *Manduca* larvae. The C18-JH equivalence per ml of the original hemolymph sample was calculated assuming an extraction efficiency of 90% (Fain, 1975).

β-ecdysone infusion

Infusions of β-ecdysone were performed on larvae paralyzed with tetrodotoxin (TTX) as described by Nijhout and Williams (1974b). Each larva was infused with 40–45 μg of β-ecdysone (as a 0.5 μg/μl solution in 10% isopropanol) over a period of 20 hours.

Parabiosis

Parabioses were performed on larvae paralyzed with 3 μg/gram body weight of TTX. Where applicable, isolated abdomens were prepared immediately prior to parabiosis. Larvae were joined at the 8th abdominal segment. Prior to the operation a ring of Tackiwax (Cenco) was applied around the area where the incision was to be made. This was necessary because wetting of the cuticle after incision made it difficult to obtain a good seal when the larvae were joined. A hole of about 4 mm in diameter was cut inside the wax ring and a few crystals of a mixture of streptomycin and reduced glutathione were placed in the wound. Two larvae were then lightly pressed together until the wax rings touched and all air bubbles had been expressed. Additional melted Tackiwax was then applied to seal the two larvae together.

Allatectomy

Allatectomies were performed as described by Nijhout and Williams (1974b). All larvae were kept without food subsequent to the operation.

RESULTS

The half-life of endogenous JH in final-instar larvae

Hemolymph JH titers were determined at various times after ligation for abdomens isolated from 5th instar larvae weighing 3.0–3.5 grams. The zero hour sample was taken from unligated larvae. The results are illustrated in Figure 1. One half hour after ligation a marked decline in JH titer had become evident, and by two hours after ligation JH was no longer detectable. The slope of the decay curve indicates that the half-life of hemolymph JH in these isolated abdomens was about 25 minutes. The JH titer in feeding larvae weighing 3.0–3.5 grams was the equivalent of 1.1×10^{-3} μg C18-JH per ml of hemolymph.

Precise quantitative determination of the JH content of blood-free carcasses proved to be impossible because the oily nature of the extract prevented the precise localization required by the scoring system of the black larval assay. Positive

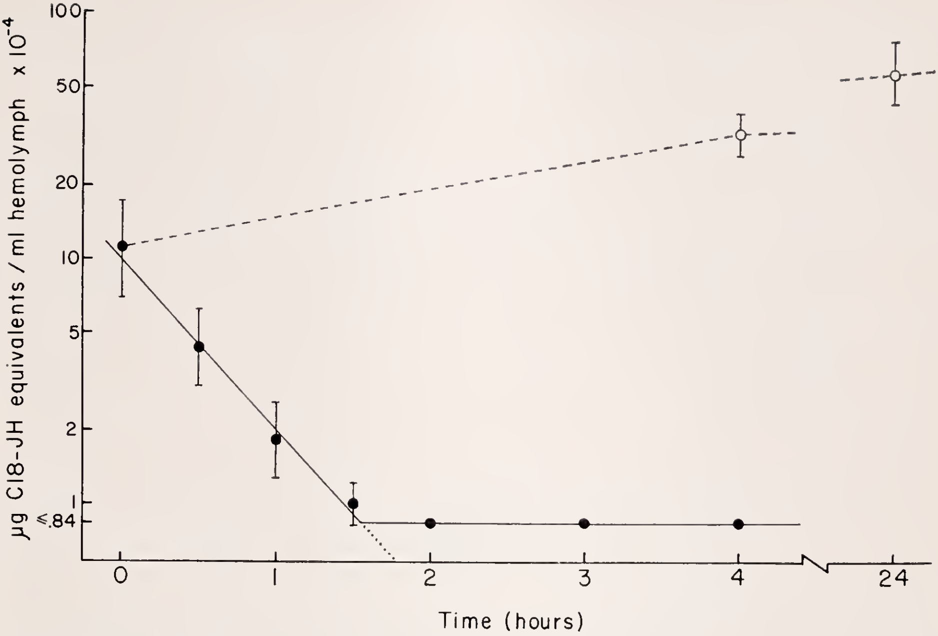

FIGURE 1. Mean JH titers in final (5th) instar larvae weighing 3.0–3.5 grams. Filled circles represent isolated abdomens ligated at 0 hours; open circles, starved larvae. The bars indicate the 95% confidence limits of the mean response of 3 replicates of 20–25 test larvae. Titers below 84 picograms/ml hemolymph cannot be detected by the assay method used. Therefore, the horizontal portion of the graph for isolated abdomens indicates undetectable JH.

scores consisting of a faint greening in the area where the assay material was applied were obtained from 86% (n = 22) and 48% (n = 21) of the assay larvae in each of two replicates with extracts of intact larvae weighing 3.0–3.5 grams. No JH was detectable in blood-free carcasses of isolated abdomens of such larvae by four hours after ligation.

When 3.0–3.5 gram larvae were starved, their hemolymph JH titers rose significantly and remained elevated for at least 24 hours (see dashed line in Figure 1). Such a rise in JH titer was not observed in feeding larvae (Nijhout and Williams, 1974b).

Persistence of JH effects after elimination of its source and in the absence of extractable JH

Nijhout and Williams (1974b) have shown that when final (5th) instar larvae of *Manduca* are induced to molt with exogenous ecdysone, the nature of their response closely reflects the titer of JH in the hemoymph. Thus, in the presence of JH, apolysis takes place initiating a larval molt. By contrast, in the absence of JH the larva is induced to expose its dorsal vessel, a prodromal symptom of pupation. In the present study it was, therefore, surprising to find that when β-ecdysone infusion of small isolated abdomens was initiated 24 hours after

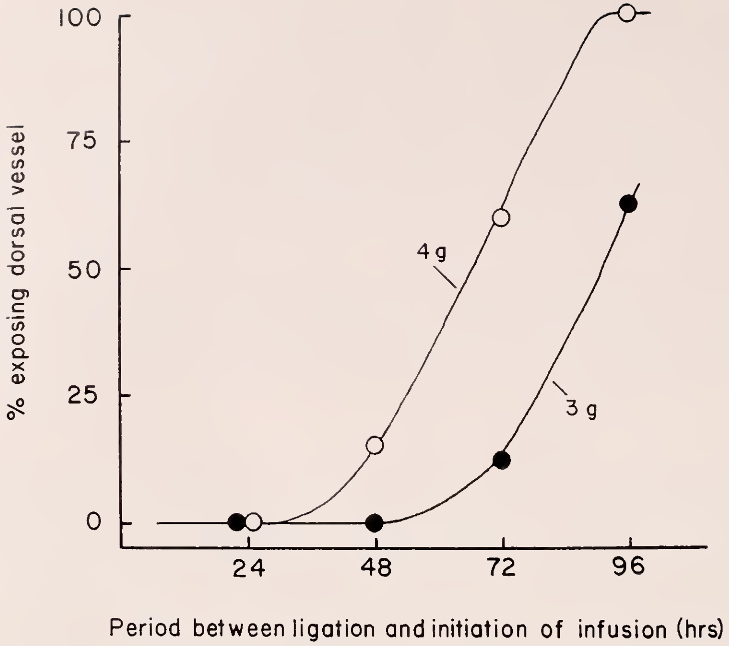

FIGURE 2. Response of isolated abdomens of 3 and 4 gram final instar larvae to infusion with β-ecdysone at various times after ligation. Each point represents 5–11 abdomens. All abdomens which did not show exposure of the dorsal vessel underwent a larval molt.

ligation, they underwent apolysis and proceeded to deposit a normal larval cuticle. Clearly, this larval response occurred long after the disappearance of JH from the hemolymph.

This phenomenon was further investigated on 5th instar larvae weighing 3.0 and 4.0 grams. Abdomens of these larvae were isolated by ligation at time zero and then stored at 25° C for 24, 48, 72 or 96 hours prior to the initiation of the β-ecdysone infusion. The results, illustrated in Figure 2, show that JH effects persisted for almost 3 days in 50% of the abdomens from 4.0 gram larvae and somewhat more than 3.5 days in 50% of the abdomens isolated from 3.0 gram larvae. It should be noted here that those abdomens that initiated a larval molt upon infusion after 72 and 96 hours did not deposit a completely normal larval cuticle. Rather, the new cuticle was smooth, yellowish, and devoid of the setae which are normally present on larval cuticle. Moreover, the spiracles of these individuals were distinctly pupal.

In 5th instar larvae of *Manduca* the presence of JH is known to inhibit the release of the prothoracicotropic hormone (PTTH). The release of PTTH that initates pupation occurs during the first photoperiodic gate after JH has disappeared from the hemolymph (Nijhout and Williams, 1974b). However, when 3.5–4.0 gram larvae were allatectomized, PTTH release occurred about a day later than would be predicted on the basis of the known period required for the clearance of JH from the hemolymph after the larva attains a weight of 5 grams (Nijhout

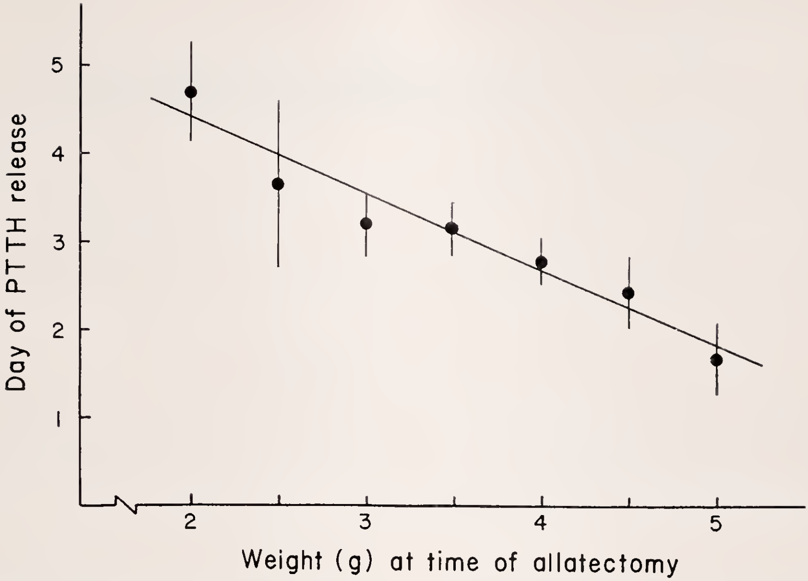

FIGURE 3. The timing of PTTH release in final instar larvae which were allatectomized at various weights and subsequently starved. The day of allatectomy is designated as day 0. The bars indicate the 95% confidence limits of the mean. Each point represents approximately 15 larvae.

and Williams, 1974b). I have investigated this discrepancy in further detail by allatectomizing larvae at various weights, isolating the resulting individuals without food, and noting the day on which PTTH release occurred. Figure 3 summarizes these experiments and shows that the release of PTTH occurred progressively later as larvae were allatectomized at lower weights. PTTH release occurred approximately 2.75 and 3.5 days after allatectomy of 4.0 and 3.0 gram larvae, respectively. These times correspond fairly well with the period required for the disappearance of JH effects in 50% of the isolated abdomens of larvae of similar weights (Figure 2).

All allatectomized larvae exposed their dorsal vessel and proceeded to deposit a perfect pupal cuticle. Forty seven percent ($n = 58$) of the starved, sham-operated control larvae weighing 3.0–4.0 grams molted to larval-pupal intermediates. PTTH release in these control larvae occurred predominantly on days 5–8 after the operation. Few sham operated larvae weighing less than three grams survived to molt, death occurring 7–12 days after the operation.

Parabiotic preparations

Intact small 5th instar larvae, weighing 3.0–3.5 grams were parabiosed to large 5th instar larvae weighing 6.8–8.0 grams. Induced by the secretion of PTTH and ecdysone of the large larva, the small partner invariably molted into a nearly perfect additional larval instar except for the absence of crochets on the prolegs and the presence of small patches of pupal cuticle on the clypeus and

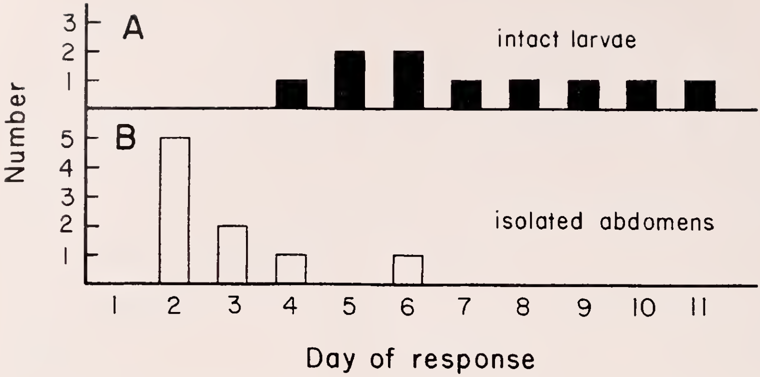

FIGURE 4. Response of final instar larvae weighing 3.0–3.5 grams to ecdysone supplied by parabiosis to 6.8–8.0 gram hormone donors. *A* shows the timing of the response of intact larvae and *B* that of isolated abdomens. *White* represents exposure of the dorsal vessel followed by pupation; *black,* apolysis and deposition of a larval cuticle. Parabiosis was done on day 0. Ligations were performed immediately prior to parabiosis.

anterior to the larval ocelli. The exposure of the dorsal vessel in the large partner and the concomitant onset of apolysis in the small partner occurred on widely scattered days (Figure 4A). This scatter was apparently due to the fact that the JH from the small larva inhibited the release of PTTH in its large partner (Nijhout and Williams, 1974b). In fact, five of ten large donors parabiosed to intact larvae showed retention of some larval characters when they pupated.

By contrast, when isolated abdomens prepared from larvae weighing 3.0–3.5 grams were parabiosed to large intact larvae, the former always exposed their dorsal vessel and proceeded to molt to perfect pupal abdomens. The exposure of the dorsal vessel occurred predominantly on day 2 after parabiosis (Figure 4B). Therefore, the abdomens had been exposed to a pulse of ecdysone on day 1, about 24 hours after the ligation. Evidently the effects of JH had been eliminated in these isolated abdomens during that 24 hour period.

DISCUSSION

The half-life of the endogenous JH activity in isolated abdomens of early 5th instar larvae is about 25 minutes. This exceedingly short half-life is probably not significantly different from the 20–30 minute half-life of exogenous (injected) JH in 5th instar *Manduca* (Ajami, 1973). This was an unexpected finding as it has heretofore been assumed that endogenous JH is somehow protected from rapid degradation, possibly by combination with one or more carrier proteins (Slade and Zibitt, 1972; Sanburg, Kramer, Kezdy, Law and Oberlander, 1975b). Protection of the endogenous JH is nevertheless evident in the abdomens of male *Hyalophora cecropia* (Williams, 1963b; Metzler, Meyer, Dahm and Roeller, 1972; Ajami and Riddiford, 1973) and possibly occurs in the earlier larval instars of *Manduca* where its half-life is longer than in the 5th instar (Fain, 1975). The half-life of JH is likely to be still shorter at a later time in the 5th instar when the titer of JH-esterase increases dramatically

(Weirich, Wren and Siddall, 1973; Sanburg, Kramer, Kezdy and Law, 1975a). Indeed, Slade and Zibitt (1972) report that JH is 85% metabolized after a 5 minute exposure to prepupal blood of *Manduca in vitro.*

Sanburg *et al.* (1975a) have recently demonstrated the existence of two classes of esterases that hydrolyse JH. General esterases are always present in the hemolymph of larvae but are capable of hydrolysing free JH only. On the 4th day of the 5th larval instar there is a sudden appearance of JH-specific esterases in the hemolymph. The latter are able to hydrolyse free JH as well as JH complexed to its binding protein. The appearance of the latter class of esterases coincides with the period of JH decline in 5th instar larvae (Nijhout and Williams, 1974b). It is of interest to note that *in vivo,* in the absence of the JH-specific esterases of Sanburg *et al.* (1975a), the half-life endogenous JH is about 25 minutes and all JH can be cleared from the hemolymph in less than two hours after its secretion ceases (Figure 1). Thus, the JH hydrolysing activity in the hemolymph of a 3 gram larva (on the 2nd day of the 5th instar) is more than sufficient to cause the elimination of JH within the 24-hour period in which it normally occurs (Nijhout and Williams, 1974b). The appearance of the JH-specific esterases on day 4 of the 5th instar does not appear to be a necessity for successful clearance of JH from the hemolymph. An alternate role for the JH-specific esterases is suggested below.

It should be understood that only a marginal amount of JH was detected in the extract from blood-free carcasses of intact early 5th instar larvae. The large amount of lipid coextracted with the JH interfered severely with the assay, even after partial purification by thin layer chromatography. It is, therefore, not rigorously excluded that the inability to detect JH in blood-free carcasses 4 hours after ligation is an artifact of the extraction and assay procedure. It is nevertheless clear that less JH is present in ligated than in intact larvae.

As shown in Figure 1, the titer of JH persisted at high levels when small 5th instar larvae were starved. Indeed, a significant increase in titer was observed, indicating that nutrition may be involved in the maintenance of proper JH titers in these larvae. When 5th instar larvae are starved at a weight of 3 grams, they eventually molt into pupae retaining many larval characters (Nijhout and Williams, 1974a; Nijhout, 1975). These results indicate that in such starved larvae the titer of JH remained elevated for at least 7–8 days.

In view of the short half-life of JH it was surprising to find that isolated abdomens underwent a perfect larval molt when infused with β-ecdysone long after JH had become undetectable in the hemolymph. This persistent effect of JH was not an artifact of the β-ecdysone infusion because an identical infusion induced normal initiation of pupation (exposure of the dorsal vessel) when given a few days after ligation (Figure 2). Furthermore, such infusions never induced larval molting when performed on intact larvae weighing 7.5 grams or more or on isolated abdomens of such larvae; that is, after JH had naturally disappeared (Nijhout and Williams, 1974b; H. F. Nijhout, unpublished results). The close correspondence between the titer of JH in the hemolymph and the response to β-ecdysone infusion demonstrated by Nijhout and Williams (1974b) indicates that when the titer of JH falls below a level detectable by the black larval assay it is also physiologically undetectable by the larva. It is, therefore, difficult to believe that the persistent effect of JH described in the present paper was due to the presence of molecular JH in the hemolymph at suprathreshold concentrations undetectable by the bioassay. Rather, it seems possible that the developmental program of the larval tissues does not require the continuous presence of JH to maintain its *status quo*. This hypothesis also finds support in the recent work of Fain and Riddiford (Harvard University, personal communication) who have shown a similar persistence of JH effects in isolated abdomens of 4th instar larvae of *Manduca* and, more importantly, in pieces of crochet epidermis cultured *in vitro* in the absence of JH (Fain and Riddiford, 1973; Fain, 1975).

The data in Figure 3 show that the ability of JH to inhibit the release of PTTH (Nijhout and Williams, 1974b) is likewise independent of the presence of molecular JH in the hemolymph. The inhibition of PTTH release was brought about by some persistent effect of JH which could remain active for 4 to 5 days in small (*e.g.* 2 grams) allatectomized larvae (Figure 3). The close agreement between the 50% points of the curves in Figure 2 and the data for the corresponding weight classes in Figure 3 show that the release of PTTH occurred precisely at the time that the persistent morphogenetic effects of JH disappeared. The effect of starvation on the release of PTTH, therefore, appears to be insignificant.

This persistent effect of JH is rather analogous to the situation found by Ohtaki, Milkman and Williams (1968) for the action of ecdysone. We are likely dealing with a fundamental principle of hormone action—namely, the fact that an overt response to a hormone is not possible until a number of biochemical events have taken place in the target tissue. The induction and completion of these unknown events have been termed the "covert effects" of the hormone (Ohtaki *et al.,* 1968) —a term which I shall adopt in this case. These covert effects presumably have a longer half-life than the hormone itself. The data in Figure 2 provide a rough estimate of this half-life as 14–20 hours.

The switchover from a larval to a pupal response upon infusion with β-ecdysone occurs at precisely the time that JH disappears from the hemolymph of normally feeding and growing 5th instar larvae (Nijhout and Williams, 1974b). In view of the short half-life of JH, it is likely that the slow decline in JH titer over the 24 hour period following the attainment of the critical weight of 5 grams (Nijhout and Williams, 1974a, b) closely reflects the secretory activity of the CA. Consequently, it appears that the CA are not instantaneously shut off when the critical weight is achieved. Rather, they begin a decline in activity at that time and become completely inactive only about 24 hours later.

The persistence of the morphogenetic effects of JH in the absence of detectable molecular JH in early 5th instar larvae (Figure 2) stands in sharp contrast to the situation later in the instar when there is a close correspondence between the actual titer of JH in the hemolymph and the response to infused β-ecdysone (Nijhout and Williams, 1974b). A mechanism must therefore exist for the rapid elimination of the covert effects of JH towards the end of the final larval instar when the CA have ceased to secrete JH. Evidence for such a mechanism was provided by the observation that late final instar larvae parabiotically joined to abdomens isolated from smaller (3.0–3.5 g) larvae were able to induce exposure of the dorsal vessel and a normal pupal molt in the latter, at a time when a simple infusion of β-ecdysone would have induced a larval molt. These results suggest that the large hormone donor supplied more than just ecdysone. It was somehow able to effect a premature elimination of the covert effects of JH in the isolated abdomens. Presumably this occurred by means of a blood-borne factor since the two partners shared a common hemolymph pool. Although there is no appreciable difference in the JH titer of larvae weighing from 2.5 to 4.5 grams (Nijhout and Williams, 1974b), Figure 3 shows the covert effects of JH are eliminated more rapidly in the latter than in the former. These results indicate that the activity of the hypothetical factor increases slowly in the course of the instar.

The possibility that the low level of ecdysone that induces the onset of the wandering stage is also responsible for the swift elimination of the covert effects of JH has been considered (Nijhout, in preparation), and it appears that ecdysone is not directly involved in this process. An attractive alternate hypothesis that could explain the experimental results discussed above presents itself on the hand of the recent publications by Sanburg *et al.* (1975a, b). It is possible that the covert effects of JH are due, not to the accumulation of biochemical effects of hormone activity as suggested above, but rather, to the persistence of tightly bound JH, undetectable by the extraction and assay procedure used. If this is the case, the blood-borne factor produced by the large parabiotic hormone donors could be the JH-specific esterases which are present in the hemolymph of these larvae. This implies that "active" JH is normally sequestered by the target tissues and the function of the JH-specific esterases (Sanburg *et al.*, 1975a, b) could be the elimination of the tightly bound JH in the target tissues rather than the hemolymph JH, the latter being efficiently eliminated even in the absence of JH-specific esterases (Figure 1).

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SUMMARY

1. The half-life of endogenous juvenile hormone (JH) in the hemolymph of early 5th (final) instar larvae of *Manduca sexta* is approximately 25 minutes. Two hours after ligation JH is no longer detectable in isolated abdomens.

2. The morphogenetic action of JH as well as its inhibitory action on the secretion of the prothoracicotropic hormone persist for several days after molecular JH has become undetectable in the hemolymph and in blood-free carcasses.

3. Evidence is presented which suggests that the action of JH is mediated by long-lived covert effects and that these covert effects of JH are swiftly eliminated toward the end of larval life, apparently through the action of an unidentified blood-borne factor.

4. Two contrasting hypotheses can explain the persistent covert effect of JH. One suggests that the covert effects are stable biochemical changes in the target tissues induced as a normal part of the molecular action of JH. Alternatively, it is possible that molecular JH persists in an unextractable form, tightly bound to the target tissues. In the latter case, the blood-borne factor that causes the elimination of the covert effects could simply be a JH-esterase.

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THE ESCAPE OF VELIGERS FROM THE EGG CAPSULES OF *NASSARIUS OBSOLETUS* AND *NASSARIUS TRIVITTATUS* (GASTROPODA, PROSOBRANCHIA)[1]

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Many species of prosobranch gastropods deposit their eggs in tough capsules affixed to hard substrates. Generally, there is a small opening near the top of such capsules, occluded by a firm plug (operculum) which must be removed before the veligers can escape. The sizeable oothecan literature deals primarily with basic descriptions—size, shape, number of eggs or embryos contained, where and when the capsules are found in the field (*e.g.,* Anderson, 1966; Bandel, 1974; D'Asaro, 1969, 1970a, 1970b; Franc, 1941; Golikov, 1961; Graham, 1941; Knudsen, 1950; Kohn, 1961; Ponder, 1973; Radwin and Chamberlin, 1973; Thorson, 1946). The remaining studies deal mostly with the structure and chemical composition of the capsules (*e.g.,* Bayne, 1968; Fretter, 1941; Hunt, 1966), rather than with how the young escape.

In a review paper on the hatching of aquatic invertebrates, Davis (1968, p. 336) suggested that the removal of the plug is usually attributable to embryonic secretion of enzymes. However, most of the ideas about how this first step in the hatching process is accomplished are without experimental support, deriving solely from descriptions of the process (*e.g.,* Bandel, 1974; Chess and Rosenthal, 1971; Davis, 1967; Houbrick, 1974; Kohn, 1961; Murray and Goldsmith, 1963; Portmann, 1955). The limited experiments which have been reported (Ankel, 1937; De Mahieu, Perchaszadeh, and Casal, 1974; Hancock, 1956; Kostitzine, 1940), deal exclusively with species that emerge from their capsules as crawling, juvenile snails. These experiments suggest that a hatching substance is produced by advanced embryos, but no attempt was made to determine the nature or properties of the substance, or the timing of its production.

The belief in a chemically-mediated release of young is not universal. West (1973, p. 4) has suggested that the capsule plug of *Colus stimpsoni* is degraded by "external factors such as bacteria and fungi"; this idea lacks any experimental support.

The egg capsules of *Nassarius obsoletus* and *N. trivittatus* are quite similar in size (approximately 1.5 mm high), number of eggs contained (forty to several hundred), and general morphology (Scheltema and Scheltema, 1964); and both have openings occluded by plugs of approximately 100 μ in thickness. Hatching takes place at the veliger stage for both species, after about one week of encapsulated development (Scheltema and Scheltema, 1964; Scheltema, 1967).

This paper demonstrates that a specific hatching substance is produced by encapsulated embryos of both species and examines some of its properties and the timing of its production, primarily for *N. obsoletus,* through laboratory experimentation.

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[1] Contribution No. 3567 from the Woods Hole Oceanographic Institution.

In addition, the actual escape of veligers from the egg capsules of *N. obsoletus* was examined. Due to the large size of the egg capsule relative to the size of an individual veliger, the small size of the opening of the capsule, and the jelly-like consistency of the intracapsular fluid (Costello and Henley, 1971), one might expect that many hours would be required for the capsules to become empty if the veligers were swimming about aimlessly. One to two days are required for all of the embryos to leave the capsules of the freshwater gastropod *Acroluxus lacustris* (Gamulin, 1973). However, if movements within the egg capsule were directed towards the opening of the capsule, much less time would be required.

I use the terminology of Giese and Pearse (1974). "Embyo" refers to an individual which has not yet taken up a free-living existence, whereas the term "larva" refers to a free-swimming individual which has entered the plankton to complete its development.

METHODS

Egg capsules were obtained from snails held in the laboratory. Specimens of *Nassarius obsoletus* were collected from the mudflats at Barnstable Harbor, Massachusetts, and specimens of *N. trivittatus* were collected by dredging in Buzzard's Bay, Massachusetts. All animals were fed on shredded clam meat.

The experiments fall into two main categories—those designed to examine the production of a hatching substance by embryos and its functional longevity, and those designed to detect its continued production by hatched larvae. Shelled, pigmented embryos were obtained from intact capsules by slicing into the capsule wall with a razor blade and expelling the embryos with gentle squeezing of the capsule using forceps. Other capsules were monitored hourly until hatching occurred; larvae from these capsules were then used at known times from the onset of hatching.

To assay for the production of hatching substance, a known number of embryos or larvae were first pipetted into the bottom of a small glass chamber, whose empty weight had been determined on a Roller-Smith Precision balance. The chamber was then reweighed and the weight difference before and after filling used to estimate the volume of water in the chamber. A freshly-deposited egg capsule was then sliced in half, parallel to its long axis, so that each half contained one part of the plug firmly held in the sectioned neck (Fig. 1). One half of this capsule was placed at the bottom of the chamber with the animals, while the other half of the same capsule was placed in a similar chamber with sea water but no embryos or larvae, as a control. Chambers were examined daily for three to six days, or until the plug was observed missing from the neck of the capsule. Chambers were held in air-tight containers at room temperature (20–23° C), at a relative humidity of 100%. Embryos and larvae remained active under these conditions for at least six days. Five-micron filtered sea water was used in all experiments.

The functional longevity of the hatching substance was assessed by placing intact egg capsules into individual glass chambers in a small volume of water (approximately 10 μl) and monitoring until hatching began. The actual volume

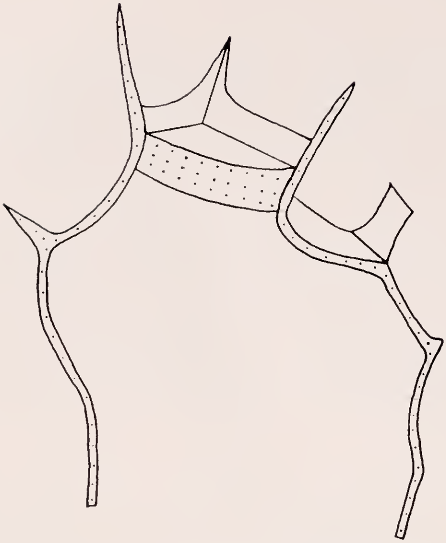

FIGURE 1. Diagramatic illustration of a *Nassarius obsoletus* egg capsule which has been sliced parallel to its long axis to expose the plug in the mouth of the capsule (drawn from a photograph). The base of the capsule has been cut away. Cut surfaces are stippled.

of water in each chamber was determined by weighing, as above. One hour after hatching had begun, the chamber fluid was removed to a new glass chamber. Fresh plug material was then added to the hatching fluid from 0 to 3 to hours later, and its integrity examined several days later. The other halves of these capsule opercula were held in sea water as controls.

Specific details for each experiment are given in the tables. In experiments testing the species specificity of hatching substance, plugs of both species were included with the advanced embryos of one species; the control plug was produced by the species whose embryos were being tested.

Additional controls were set up to examine the possibility of eventual spontaneous plug detachment. Capsules containing osmotically-killed eggs and embryos were held for two months in five-micron filtered sea water at room temperature; periodically these were gently squeezed to assess plug integrity. Freshly deposited egg capsules were emptied of their contents and sliced in half as described above, and their opercula examined after two months in sea water at room temperature.

To compare experimentally-determined densities of embryos required to initiate plug release with the actual density of embryos in egg capsules, the volumes of four *N. obsoletus* capsules which had hatched out known numbers of larvae were estimated with a microsyringe.

An equation predicting the rate of collisions of confined gas molecules with the walls of their container was used to predict the rate at which veligers would leave an egg capsule if their escape were a purely chance process. The equation is (Sears, 1959): number of collisons per unit area per unit time $= \frac{1}{4}\ n\ \bar{v}$, where "n" is the number of molecules per unit volume, and "$\bar{v}$" is the average speed of the molecules in the container. The equation assumes that the gas molecules are uniformly distributed in the container. By treating the encapsulated veligers as gas molecules and assuming that the collison of a veliger with the opening at the top of the capsule results in the escape of the individual from the capsule, the rate at which veligers should leave is predicted by multiplying the above expression by the surface area of the opening of the egg capsule, 0.08 mm^2 ($N = 2$). The average speed of encapsulated veligers was determined by analyzing movie footage of the movements inside an emptying egg capsule when only 8 veligers remained, by which time individual movements could be followed. The model egg capsule was assumed to initially contain 50 veligers. The value of "n" in the equation was changed after each hypothetical 5-minute interval by subtracting the number of escapees in the interval from the number present at the beginning of the interval and dividing by the hypothetical egg capsule's volume, 0.6 μl. Predicted rates of escape were compared with those actually observed in the laboratory.

RESULTS

From the time the capsule is deposited until just prior to hatching, the capsule plugs of both species have very distinct outlines and are firmly held in the necks of the egg capsules (Fig. 1). The plugs do not actually dissolve in the hatching process, but become amorphous, greatly softened and are easily dislodged from the capsule wall. Plug degradation seems to be a sudden event, rather than a gradual process.

No control plugs became softened or loose in the necks of their egg capsules over the two-month observation period or in the course of any particular experiment. Those plugs destined to be degraded generally fell away from the necks of the sliced egg capsules by the day following the start of the experiment. This supports the contention that degradation of the plug does not involve a gradual build-up of hatching substance within the capsule.

The results of 14 experiments in which different numbers of embryos were confined in volumes of sea water ranging from 2.4 μl to 230.0 μl are presented in order of increasing embryonic density (Table I). Plug release was scored in all chambers holding more than 0.4 embryos per μl, regardless of the absolute number of individuals contained.

Although no clear cut-off time is revealed, the secretion of hatching substance is no longer detectable four hours after hatching for *N. obsoletus*, and by 24 hours after hatching for *N. trivittatus* (Table II). Indeed, production by *N. obsoletus* larvae seems to decline within one hour of hatching, since plugs were degraded only at the highest densities of one-hour old larvae. Note that all larval densities used in these experiments were well above those previously found adequate for plug dissolution by embryos (Table I). The hatching substance is thus no ordinary metabolite, but rather a substance produced specifically to loosen the capsule plug prior to escape of the veligers. Larvae which have apparently ceased production of hatching substance do not degrade capsule opercula, indicating that physical manipulation does not play a major role in removing the plug.

TABLE I

Production of hatching substance by advanced embryos of Nassarius obsoletus. *Plus represents release of plug from capsule neck; minus represents plug remaining firm in capsule neck.*

Number embryos	Number embryos per μl	Outcome
45	0.196	−
55	0.239	−
2	0.400	−
4	0.408	+
210	0.913	+
6	1.000	+
16	1.176	+
45	1.364	+
16	1.454	+
329	1.645	+
16	1.951	+
13	2.241	+
13	2.500	+
16	4.473	+

The hatching substance produced by *N. obsoletus* embryos loses its potency within 3 hours in sea water at room temperature (Table III).

Despite the similarity between the capsules of the two species examined, the substance produced by the embryos of one species is incapable of degrading the plugs of the other (Table IV).

TABLE II

Production of hatching substance by larvae of N. obsoletus *and* N. trivittatus (*) *at various times after the initiation of hatching. Plus represents release of plug from capsule neck; minus represents plug remaining firm in capsule neck.*

Hours after initiation of hatching	Number larvae	Number larvae per μl	Outcome
1	14	2.641	−
1	15	3.750	−
1	14	3.784	+
1	20	4.545	+
2	19	3.518	−
2	31	5.000	−
2.5–3	24	3.750	+
3.5–4	14	2.258	−
4	93	1.390	−
4	17	3.696	−
5	19	2.714	−
8	17	7.083	−
8	14	3.500	−
11	8	3.077	−
16	19	4.750	−
24–28	177	1.264	−
* within 24	194	1.492	−
* 24–48	217	1.447	−

TABLE III

Functional longevity of N. obsoletus *hatching substance. Plus represents release of plug from capsule neck; minus represents plug remaining firm in capsule neck.*

Number larvae hatched	Number larvae per µl	Hours between removal of hatching water and addition of plug	Outcome
45	6.618	0	+
49	7.000	0	+
25	4.630	0	+
38	4.130	0	+
34	3.840	1.50	+
41	4.254	2.25	−
44	4.889	2.50	+
51	6.892	3.0	−
47	5.014	3.0	−

The average speed of veligers within the capsule when only a few remained was 1.8 mm per minute (N = 11 measurements on 8 individuals). Using this value of $\bar{v}$, the equation predicts that 98% of the veligers will leave the capsule within only 55 minutes. Both of the capsules actually monitored, containing 45 and 51 individuals, lost 98% of their veligers within 45 to 50 minutes after hatching commenced, and the shape of the escape curve is similar to that predicted for a chance escape (Fig. 2). Thus, movements within the capsule are probably undirected. The observed rate of escape is initially in better agreement with that predicted for a slower swimming speed instead of the speed actually determined (Fig. 2). This is perhaps attributable to a decrease in the viscosity of the intracapsular fluid during hatching, since the fluid is soluble in seawater (Costello and Henley, 1971).

DISCUSSION

The mean number of developing eggs enclosed by a single capsule is highly variable between species, even within the same genus. *Nassarius vibex* capsules,

TABLE IV

Species specificity of hatching substance produced by veligers of N. obsoletus *and* N. trivittatus. *Plus represents release of plug from capsule neck; minus represents plug remaining firm in capsule neck.*

Species	Number embryos	Number embryos per µl	Outcome	
			N. obsoletus	N. trivittatus
N. obsoletus	291	1.455	+	−
N. obsoletus	264	1.600	+	−
N. obsoletus	19	3.800	+	−
N. obsoletus	23	4.600	+	−
N. trivittatus	208	3.480	−	+
N. trivittatus	224	1.600	−	+

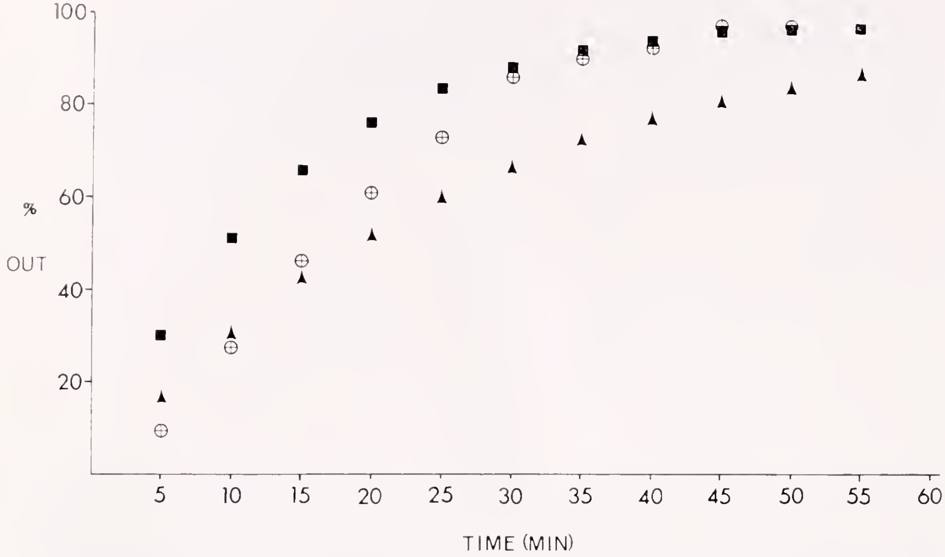

FIGURE 2. The observed and predicted rates of escape of veligers from egg capsules of *N. obsoletus.* Squares trace the predicted rate of escape based on the average swimming speed measured, 1.8 mm per minute. Triangles trace the predicted rate of escape based on a swimming speed of 1.0 mm per minute. Circles trace the average observed rate of escape from two capsules.

for example, generally hold 20 eggs (Scheltema, 1962), *N. pygmaeus* capsules hold 40 to 50 eggs (Lebour, 1937), and *N. obsoletus* capsules hold as many as 300 eggs (Costello and Henley, 1971), although Scheltema (1962) gives a range of 40 to 150. If removal of the capsule operculum is generally a chemical process, one might expect the parcelling of eggs into capsules to be related to the minimum number of individuals required to produce sufficient hatching substance to degrade the plug. However, the actual densities of embryos in *N. obsoletus* capsules are over one hundred times the minimum density required for successful hatching (Table V). For these capsule volumes (Table V), a single individual should be able to remove the operculum and escape.

Hancock (1956) and Kostitzine (1940) implied that the loosening of the operculum is a gradual process in *Urosalpinx cinerea* and *Purpura* (= *Nucella*)

TABLE V

Embryos per μl in egg capsules of N. obsoletus.

Capsule height mm	Number embryos contained	Approximate capsule volume (μl)	Approximate number embryos per μl
1.25	47	0.6	78
1.4	51	0.6	85
1.8	152	1.8	84
1.3	17	0.5	34

lapillus capsules, respectively. The only detailed, relevant experimental work done on marine invertebrate hatching is that investigating the dissolution of sea urchin fertilization membranes prior to gastrulation. As in *N. obsoletus,* the substance produced by embryos of the urchin *Strongylocentrotus pulcherrimus* is apparently produced in a short pulse. There is evidence of hatching substance production between 11 and 14 hours after fertilization, but not before or after this period (Sugawara, 1943).

In contrast to the hatching substance produced by *N. obsoletus,* that produced by the urchin *Arbacia punctulata* is said to remain active for several weeks in sea water, although the conditions of storage are not given (Kopac, 1941). Functional longevity of the hatching substance has not been specifically considered for other species studied, although the substance produced by the urchins *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina* was concentrated for experiments by evaporating the hatching water for 3 to 10 hours at room temperature, after which time it was quite potent (Yasumasu, 1960).

The species specificity of the hatching substance produced by the two *Nassarius* species in my study contrasts sharply with the known action of other marine invertebrate hatching substances. Those produced by the urchins *Strongylocentrotus purpuratus* and *S. franciscanus* will dissolve the fertilization membranes of either species (Barrett and Angelo, 1969). In the Ascidiacea, embryos also dissolve away their fertilization membranes. There is no evidence of species specificity of the hatching substance in the five species studied. In fact, the substance produced by *Ascidia conchilega* acts more quickly on the chorion of *Phallusia mammillata* eggs than on the chorion of its own eggs (Berrill, 1929)!

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SUMMARY

1. The loosening of the egg capsule plug prior to escape of the veligers is shown to be chemically mediated in *Nassarius obsoletus* and *N. trivittatus*.

2. The hatching substance is not produced continuously during development, but rather in a short pulse beginning just prior to hatching and ending within 4 hours of escaping from the capsule, for *N. obsoletus*.

3. The hatching substance produced by the embryos of one species is effective only on the capsule plugs of that species, for the two species studied.

4. The substance is functionally short-lived, at room temperature in sea water, losing its potency within three hours after its secretion by *N. obsoletus*.

5. The observed rate at which *N. obsoletus* veligers leave their egg capsules is shown to be in close agreement with the rate predicted from an equation assuming random movements of individuals within the capsules.

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STAGES IN THE LIFE HISTORY OF A SYMBIOTIC ZOOXANTHELLA IN PELLETS EXTRUDED BY ITS HOST *AIPTASIA TAGETES* (DUCH. AND MICH.) (COELENTERATA, ANTHOZOA)

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In 1944 Kawaguti extracted from the coral *Acropora corymbosa* a number of its symbiotic algae and grew these in culture. Observation of these cultures revealed that the zooxanthellae regularly gave rise to motile individuals, and from their characteristics he classified them as being dinoflagellates of the Genus *Gymnodinium*. McLaughlin and Zahl (1957, 1959, 1966) subsequently extracted symbiotic zooxanthellae from a number of coelenterates and produced axenic cultures of these algae. The coelenterates used were the scyphozoan *Cassiopeia sp.,* the alcyonarians *Antillogorgia acerosa* (Passas) and *Plexaurella dichotoma* (Esper), the scleractinians *Cladocora sp.* and *Porites sp.* and the actinian *Condylactis sp.* The algae isolated from all these species appear to be the same insofar as they all gave rise to similar gymnodinoid forms.

Freudenthal (1962), using axenic cultures of zooxanthellae obtained from *Cassiopeia,* also identified this symbiont as being a dinoflagellate and assigned to it the name *Symbiodinium microadriaticum* (Freudenthal). He described the life history of the organism and found it to be a cycle in which the zooxanthellae are encysted stages that alternate with motile ones. Taylor (1971a) later renamed the species *Gymnodinium microadriaticum* (Freudenthal) and in subsequent work (1973a) described it as being the dinoflagellate symbiont most commonly occurring among benthic dwelling hosts. Based on an extended study of this species, both *in situ* and in culture, he further proposed a modified version of Freudenthal's scheme as being a more accurate representation of the life history of that organism (see Fig. 1).

The immature, mature, dividing and degenerate cysts which form stages in the life history of *G. microadriaticum* have all been observed in mucous boli and strings extruded under normal conditions by a variety of coelenterates (Taylor, 1969, 1973a; Goreau, Goreau, Yonge and Neumann, 1970; Reimer, 1971; Yonge, 1973; Trench, 1974). However, with the exception of the dinoflagellate *Amphidinium klebsii* (Kofoid and Swezy) which is known to be both free-living and symbiotic (Taylor, 1971b) and the motile stages of *S.* (= *G.*) *microadriaticum* which were observed by McLaughlin and Zahl (1959) to be released from disintegrating specimens of *Condylactis* kept in an aquarium, dinoflagellate symbionts have not been collected as free-living stages in the environment adjacent to their hosts, although T. F. Goreau (N. I. Goreau, Zoology Department, University of the West Indies, personal communication) had recorded the presence of dumb-bell shaped zoospores among zooxanthellae freshly extracted from *Zoanthus* sp. in 1965.

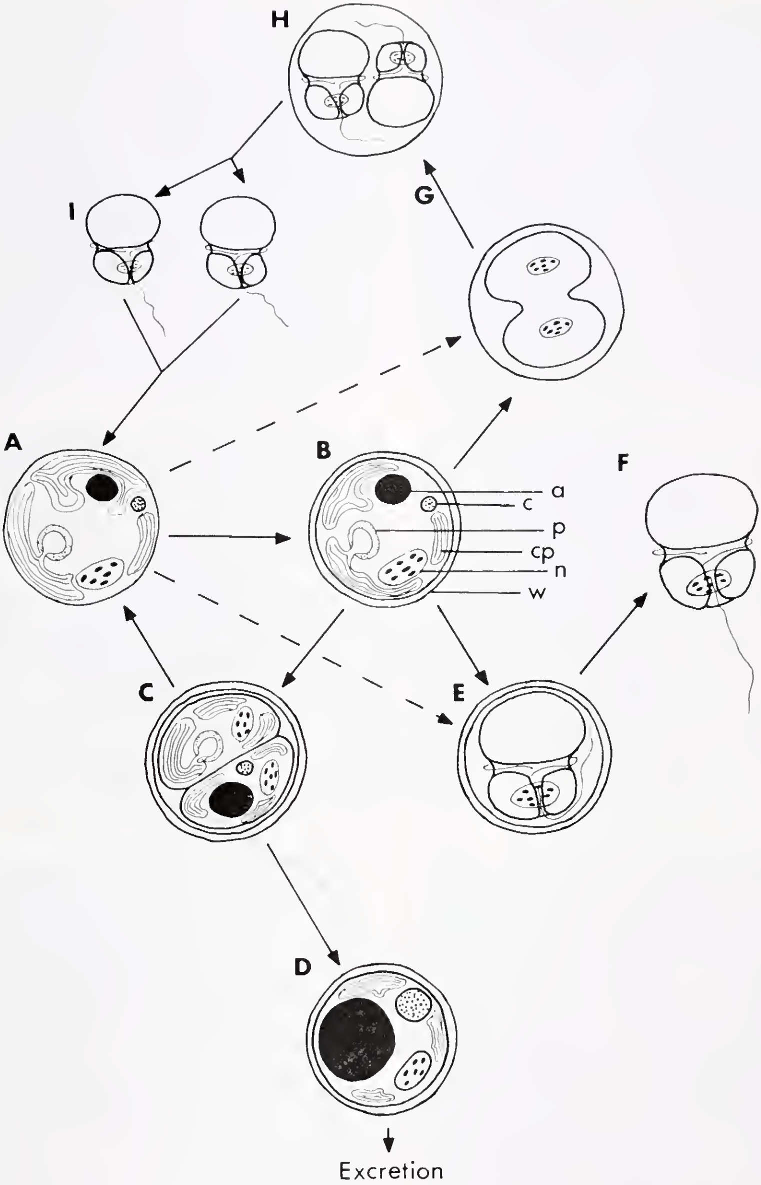


FIGURE 1. Outline of the life history of *Gymnodinium microadriaticum* after Taylor (1973a). (A) shows an immature cyst; (B) a mature cyst; (C) division of a cyst into a physiologically younger cell which reverts to an immature cyst (A), and a physiologically older cell (D) which degenerates and is excreted. (E) shows a zoosporangium; (F) a motile zoospore; (G) and (H) the development of gametes and (I) the release of mature gametes. The accumulation body is represented by (a); calcium oxalate (c); chloroplast (cp); nucleus (n); pyrenoid (p); and cyst wall (w).

This paper reports the observation of a number of stages in the life cycle of the zooxanthella symbiotic with the anemone *Aiptasia tagetes* in material extruded by the host.

OBSERVATIONS

Healthy specimens of *Aiptasia tagetes* kept in the laboratory regularly extrude dark-brown, compact pellets made up almost exclusively of zooxanthellae held together by mucus. These pellets are discharged separately from those containing faecal material (R. D. Steele, unpublished observation). Examination of smears of these algal-containing pellets under the microscope revealed the presence of the following stages in the life cycle of the alga.

Stages which are always observed

Cysts (see Fig. 2A and B). These form the majority of cells in the pellets. They are 5.3 to 11.7 microns in diameter, show a pyrenoid, nucleus, vacuoles with rapidly moving contents and often a reddish-brown accumulation body. Mature cysts all have a thick wall (see Fig. 2B).

Dividing cysts (see Fig. 2C, D, E). The number of these in a pellet varies widely but never exceeds 25 percent of the total. Normally, two closely apposed daughter cells are present within a cyst wall but quite frequently groups of four have been noted (see Fig. 1A). In a number of cases the cyst wall has been lost. This occurs among zooxanthellae in which division is complete and whose daughter cells, though still attached, have developed their own cell walls (see Fig. 1D and E).

Degenerate cysts (see Fig. 2F). These cysts form a small percentage of the total algal content of the pellets. Variable degrees of degeneracy are found, but in general the degenerate zooxanthella is characterized by the presence of a large accumulation body, an increase in number and size of cytoplasmic inclusions, and a reduction in chloroplast size.

Stages which are occasionally observed

Zoosporangia (see Fig. 3A, B, C). These may constitute up to 20 percent of the algal cells in some pellets. They are of the same dimensions as the other zooxanthellae and the cytoplasm contains inclusions, a pyrenoid and almost invariably an accumulation body. Each zoosporangium consists of a cyst wall enclosing a zoospore inside. This organism is released and becomes motile when the cyst wall ruptures (Fig. 3C).

Motile zoospores (see Figs. 3 D–F; 4 A–E). When present these cells may constitute up to 25 percent of the total algae in a pellet.

Structure

Zoospores are about the same size as vegetative cysts and ordinarily are somewhat dumb-bell shaped with no cyst walls. The wider anterior region (= the epicone) always contains a relatively large accumulation body and is separated from the narrower posterior region (= the hypocone) by a transverse groove which runs

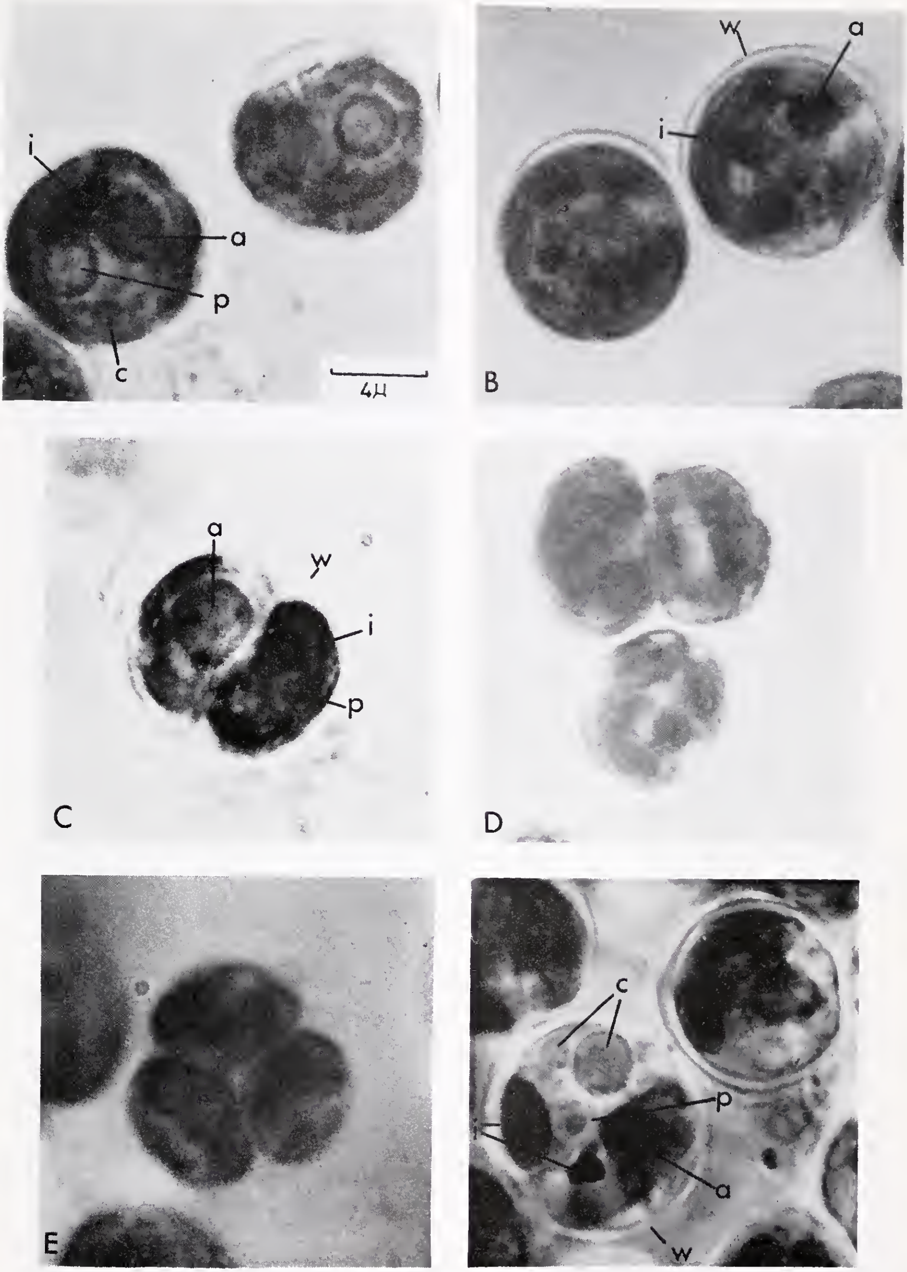
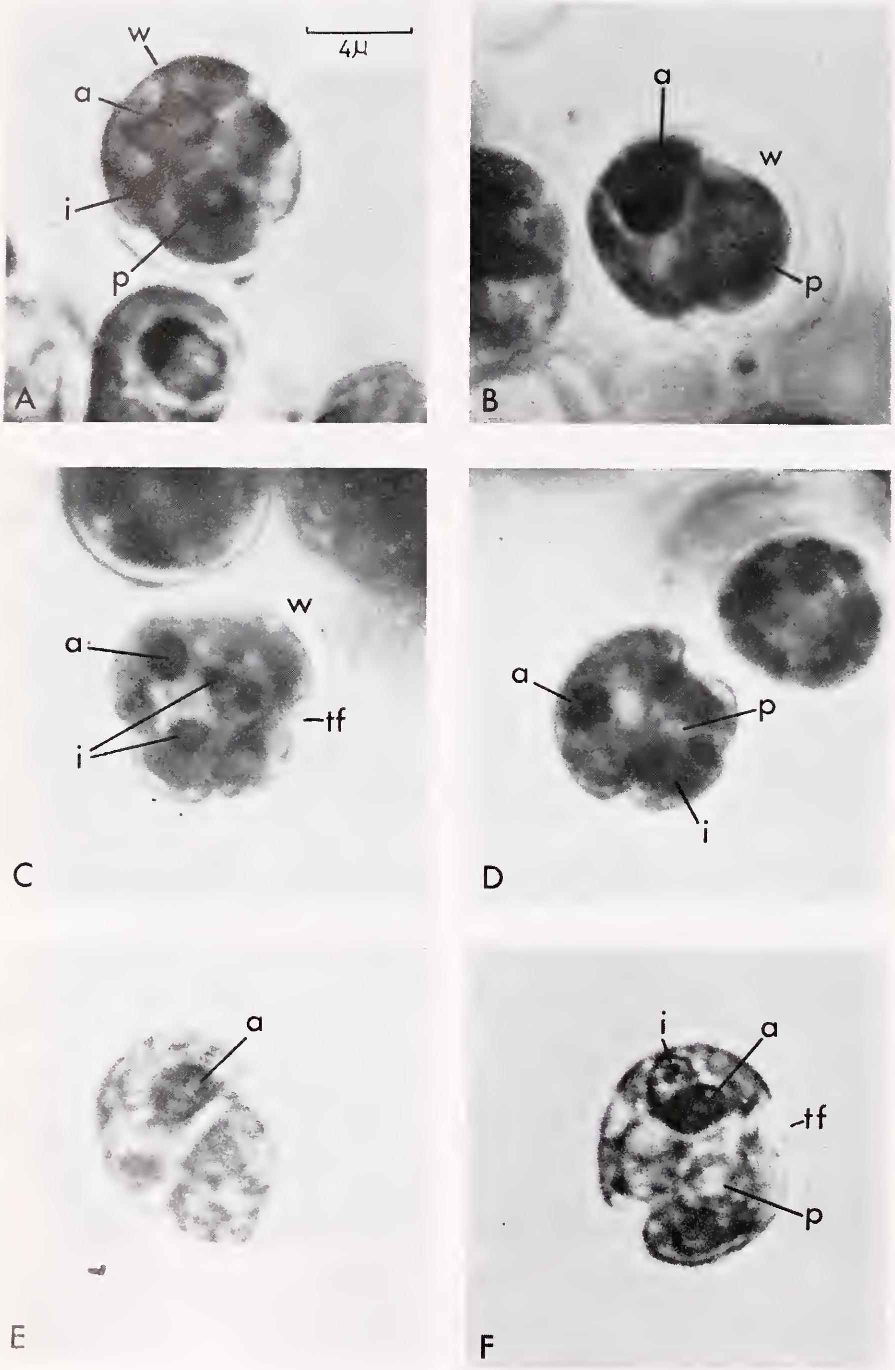


FIGURE 2. Photographs of various stages in the life history of the zooxanthella from *A. tagetes*. (A) shows immature cysts; (B) mature cysts (note cell walls); (C) a dividing cyst; (D) a dividing cyst in which the parent wall has been lost; (E) a cyst dividing into four daughter cells, and (F) degenerate cysts. Inclusions are represented by (i); other symbols are as in Figure 1. The 4 micron scale-bar in (A) is applicable to all six photographs.



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around the organism and in which lies a flagellum. There is a depression at the tip of the posterior end which leads into a groove (= the sulcus) along one side of the organism which runs up to the transverse groove (see Fig. 3D). A long, posteriorly-directed flagellum extends from this groove and is about twice the length of the cell body (see Fig. 4C). All zoospores have more or less the same shape, but as they undergo contractions during swimming, there is variation from individual to individual.

Apart from the already mentioned accumulation body, chloroplasts and a pyrenoid are usually recognizable and in many cases other inclusions are also identifiable.

Locomotion

Two types of movements were displayed by these zoospores, each type alternating with the other at random intervals of time.

Forward movement (see Figs. 4C and 5). This type of movement is displayed when the organism darts rapidly from place to place with the anterior end foremost. Propulsion in these cases is supplied by the posterior flagellum and the organism rotates on the axis of this flagellum at the same time as it moves forward.

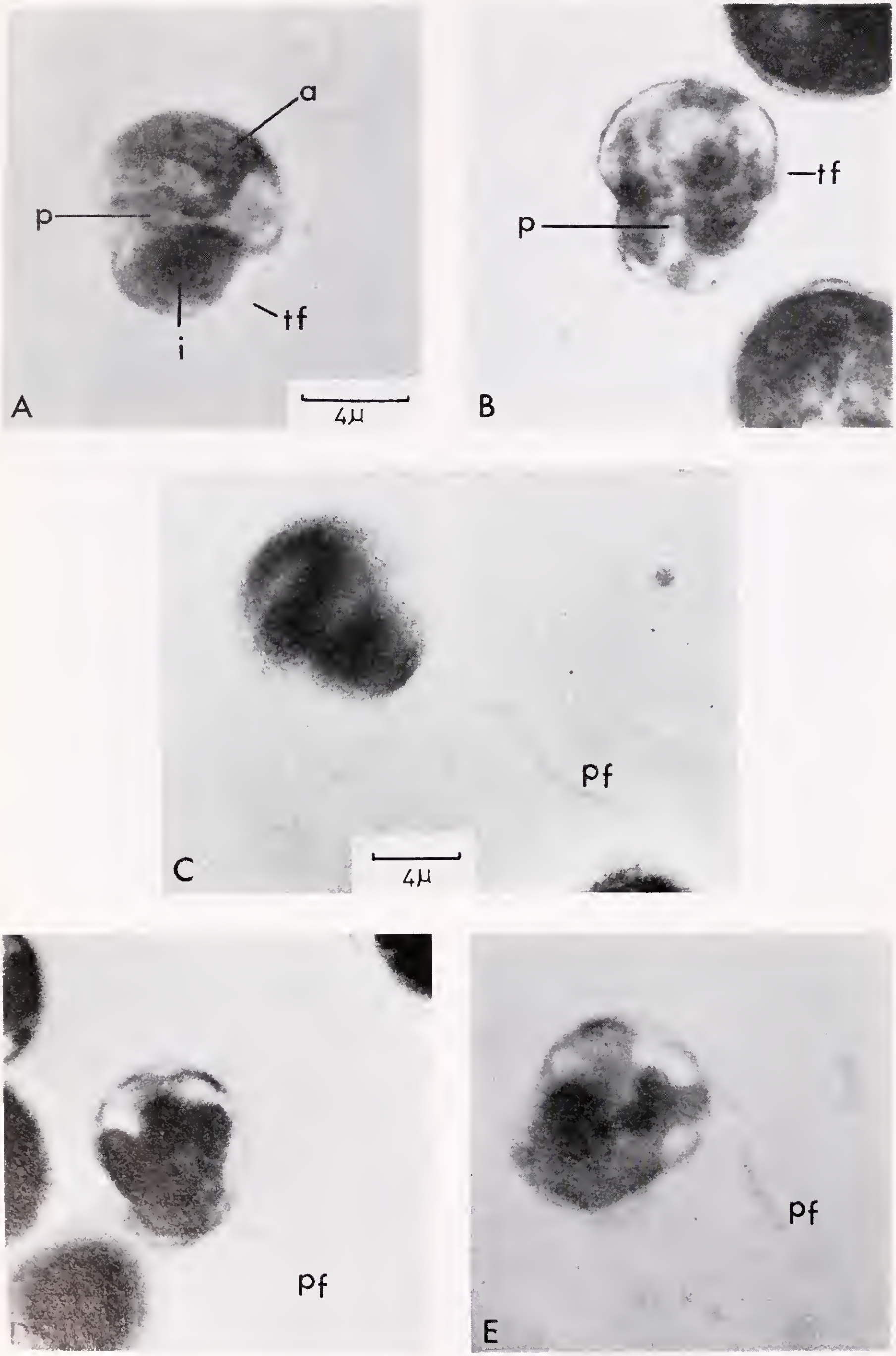
Gyratory movement (see Fig. 5). In order to carry out this type of movement, the zoospore first attaches itself to the substratum by means of the tip of the posterior flagellum. It then positions the cell body almost at right angles to the flagellum and gyrates. The gyrations are apparently produced through the action of the transverse flagellum. Individuals appear to gyrate to the right or to the left at random, and some have been observed to gyrate in one direction and then do so in the opposite direction immediately afterwards. The average rate of gyration is 3.3 evolutions per second at a room temperature of 21° C and zoospores have been observed to keep up one spell of gyrations for as much as 6½ minutes.

A number of these zoospores have been observed to stop swimming whereupon they immediately cast off their flagellae (see Fig. 4D and E) and become indistinguishable from the surrounding zooxanthellae.

DISCUSSION

The algal forms described in this paper are quite clearly stages in the life history of the zooxanthella symbiotic with *A. tagetes*. Figure 6 shows drawings of the identified stages arranged to show the likely relationship which they have with one another. Comparison of this outline with that of Taylor (1973a) (see Fig. 1) shows that probably only the sexual stages of the life cycle of this zooxanthella have not been found. Some of these algal stages resemble those of *S.* (= *G.*) *microadriaticum* shown in the photographs of McLaughlin and Zahl (1957) and Freudenthal (1962) as well as those shown in the diagram of the life cycle of *G. microadriaticum* outlined by Taylor (1973a). However, the zoospores

FIGURE 3. Photographs of various stages in the life history of the zooxanthella from *A. tagetes*. (A) and (B) show zoosporangia; (C) a zoospore breaking out of its cyst wall; (D), (E) and (F) zoospores. The transverse flagellum is represented by (tf); other symbols are as in Figures 1 and 2. The 4 micron scale-bar in (A) is applicable to all six photographs.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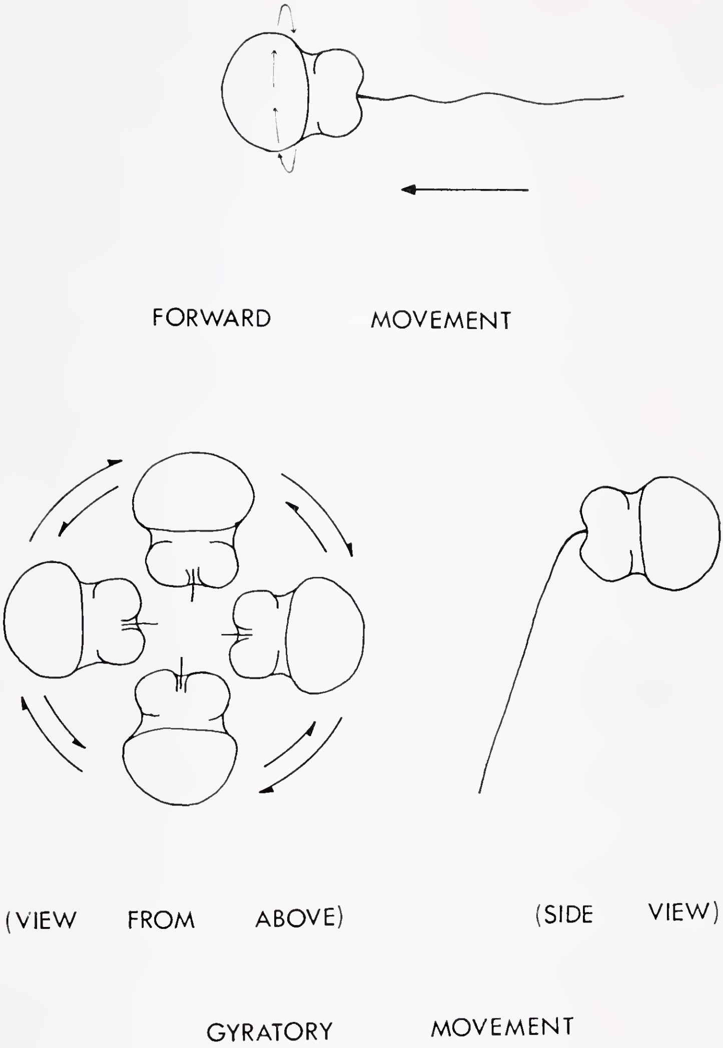

FIGURE 5. Diagrams showing the two types of movement carried out by zoospores of the zooxanthella of *A. tagetes.*

figured here resemble those obtained from *Acropora* by Kawaguti (1944), and differ from those described by McLaughlin and Zahl (1957) and Freudenthal (1962) in containing a relatively large reddish-brown accumulation body, which

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FIGURE 4. Photographs of various stages in the life history of the zooxanthella from *A. tagetes.* (A) and (B) represent zoospores, showing the transverse flagellum; while (C) represents a swimming zoospore, showing the long posterior flagellum. The organism is rotating about the axis of this flagellum as it moves forward. (D) and (E) show successive stages in the release of its flagella by a zoospore which has just stopped swimming. The posterior flagellum is represented by (pf), other symbols are as in Figures 1–3. The 4 micron scale-bar in (A) is also applicable to (B) and (E); the 4 micron bar in (C) also to (D).

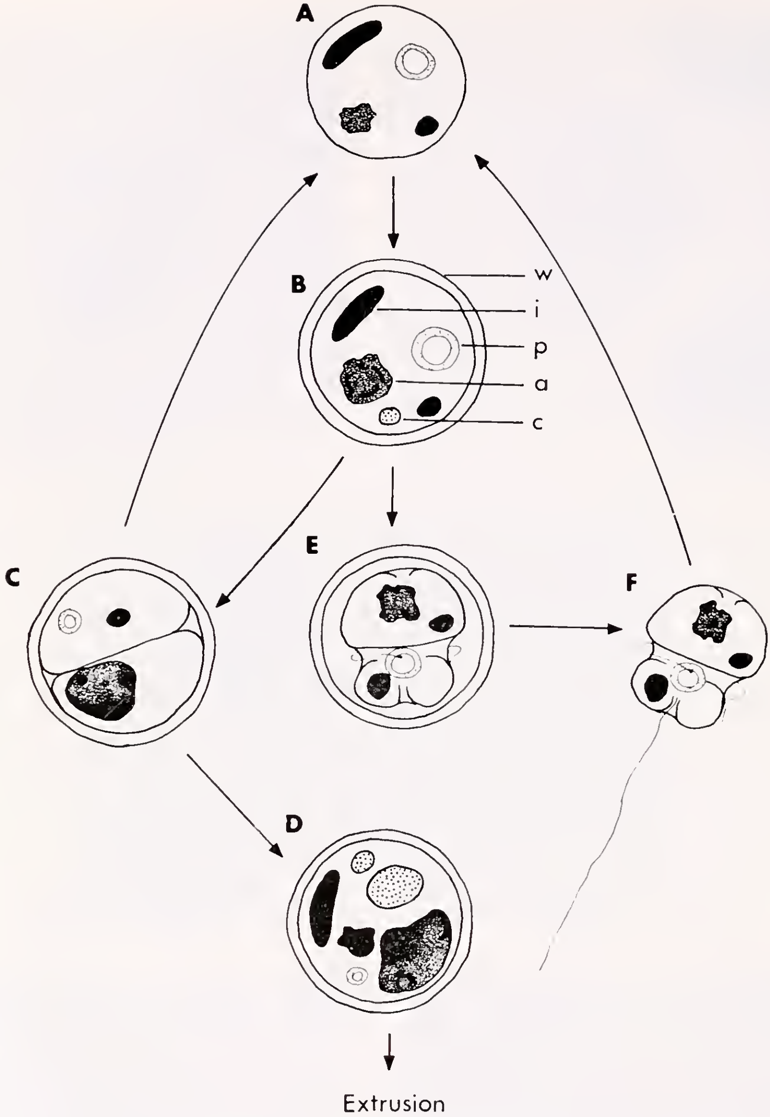

FIGURE 6. Outline of the likely relationships which exist between the stages in the life history of the symbiotic zooxanthellae from *A. tagetes* which have been identified in pellets extruded by the host. Chloroplasts are not drawn in. Symbols are as in preceding figures.

according to Taylor (1973b) is apparently a feature found only in the non-motile vegetative stages of dinoflagellate symbionts.

The irregular occurrence of an accumulation body in the zoospores referred to suggests either that the algae concerned belong to different strains—an idea for which there is some evidence (McLaughlin and Zahl, 1959; Trench, 1971a, b;

Kinzie, 1974)—or that cysts of any age may give rise to zoospores; young cysts thereby produce forms without accumulation bodies while older cysts which already possess such granules retain them when they undergo transformation into motile forms. Also, conceivable differences between the environments in which zoospores develop may cause the presence or absence of the accumulation body. Whether one or a combination of these is the correct answer is not known, but work on this problem is continuing.

The ejection of healthy symbiotic zooxanthellae by their hosts has been observed to occur as a normal process in *Zoanthus danae* (Le Conte) and *Zoanthus sp.* (Reimer, 1971), in *Palythoa sp.* (Trench, 1974) and in *A. tagetes* (Steele, present communication); and these new observations of motile zoospores in pellets extruded by *A. tagetes* serve to further underline the point that coelenterate hosts may release viable symbionts in this way. In addition, the fact that the motile forms have been found outside the host animal raises for the first time the definite possibility that under normal conditions a symbiotic zooxanthella could be transmitted from one host coelenterate to another by means of a motile stage in the alga's life history. Kinzie (1974), using cultures of dinoflagellates from various hosts, demonstrated that aposymbiotic specimens of the gorgonian *Pseudopterogorgia bipinnata* (Verrill) can be infected only by the zoospores of *G. microadriaticum* and not by the cysts; and he has also shown that during the process of infection the motile algae were attracted by and swam towards the gorgonian polyps.

On the basis of Kinzie's evidence, therefore, it seems very likely that zoospores released by *A. tagetes* would swim towards and infect any appropriate host which happened to be available. However, it must be pointed out that the existence of an indirect route for the transmission of symbionts, in which the released zooxanthellae are first engulfed by small organisms which in turn are captured and eaten by the host animal, is a distinct possibility. Evidence for this second route is to be found in the observation that zooxanthellae extruded by *A. tagetes* are frequently engulfed by ciliates of various kinds which are present in the pellet (R. D. Steele, unpublished observation).

The observed metamorphosis of zoospores into the vegetative cells takes place very rapidly, beginning in each case with the loss of the flagella followed by rounding up of the cell body. This process has also been described in *G. microadriaticum* both by McLaughlin and Zahl (1957) and Freudenthal (1962) and is obviously the norm among symbiotic zooxanthellae. The presence of behavior of this type in these motile forms suggests that the zoospore is definitely the dispersal stage of the alga whose mobility is lost once the process of transmission to another host has been accomplished. As to why both zoosporangia and zoospores are present in the extruded pellets of *A. tagetes* on some occasions only is not known, but work is continuing on this aspect of the subject.

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SUMMARY

1. Various morphological stages in the life history of the zooxanthella symbiotic with *Aiptasia tagetes* have been identified in pellets extruded by the actinian host. One of the stages is a motile zoospore.

2. This zoospore resembles that observed by Kawaguti (1944) and differs from those identified in axenic cultures of *G. microadriaticum* in containing a large accumulation body. Suggestions are made as to the significance of this difference.

3. It is very likely that the zoospore is an infective stage in the life history of the zooxanthella.

4. There is some evidence to suggest that indirect infection of hosts by zooxanthellae is also possible.

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INDEX

A

Acclimation, in marine snails, 331
Accumulation body, in zooxanthella, 590
Acid phosphatase, in larval diapause, 151
Active zones, in *Torpedo* synapses, 429
Activity, sideral variation in, *Mus musculus,* 128
  diel rhythms, in bluegill, 96
Adair, W. S. and J.-P. Revel. Membrane changes associated with the early stages of cell fusion in *Tetrahymena,* 419
Aedes aegypti, sexual behavior in, 365
A. taeniorhynchus, egg production in, 384
Aequorin luminescence, and calcium, in amoebae, 448
Age-dependent, alleles, in *Modiolus,* 446
Aggregation factor, in *Microciona,* 419, 430
Aggressive behavior, in hermit crabs, 205
Alleles, in *Modiolus,* 446
Allen, R. D. See D. L. Taylor, 448
Alveoli, cytoplasmic, 551
Amino acids, and feeding behavior, 522
δ-aminolevulinic acid, in *Cyanidium caldarium,* 432
Aminopyridine, action on synaptic transmission in squid, 435
Amoebae, calcium detection in, 448
Anderson, D. See T. Humphreys, 430
Anderson, J. M. Ultrastructure of the extracellular matrix around dissociated sponge cells, *Microciona prolifera,* 419
Anderson, P. A. V., and J. F. Case. Electrical activity associated with luminescence and other colonial behavior in the pennatulid *Renilla kollikeri,* 80
Angler-fish, proglucagon to glucagon in, 439
Anthozoan colonies, behavior in, 80
Antigrowth action, of tryptophan photoproduct, 452
Arbacia, ciliary length in, 444
  embryos, spicule formation in, 439
  histone acetylation in, 447
  larval spicule, crystal property of, 431
Arbacia eggs, potassium exchange in, 422
  respiration in, 441
  sperm block in, 449
  ultrastructure of, 446
Arenicola cristata, urease from, 424
Armadillidium vulgare, ultrastructure of, 467
Arsenazo III, in liposomes, 450
  in squid axon, 421
  on *Limulus* photoreceptors, 449
Arctic algae, 408
Ascidian eggs, differentiation of enzymes in, 451
Assay, JH, in *Manduca sexta,* 568
Autogeny, in salt marsh mosquitoes, 384
Axon, giant, squid, 421
  medial, in *Homarus,* 428
  squid, birefringence, dichroism in, 444
Azariah, J., M. M. Ismail and M. A. Najib. Investigation on the ecology and respiratory responses of the Hemichordate *Ptychodera flava* to tidal cycles and salinity changes, 455

B

Balanus amphitrite, tissue cultures of, 316
B. eburneus, tissue cultures of, 316
B. improvisus, tissue cultures of, 316
Barkalow, D. and A. Farmanfarmaian. Energy coupling mediated by a brush border Na, K-ATPase in the active absorption of glycine in toadfish intestine, *in vivo,* 420
Barker, J. L. See H. B. Pollard, 442
Bartolf, M. M. See J. E. Jurgenson, 432
Bauer, G. E. See B. D. Noe, 439
Beale, S. I. See J. E. Jurgenson, 432
Behavior, in *Melampus,* 442
  in *Proboscidactyla flavicirrata,* 236
  thermoregulatory in bluegills, 96
Behavioral electrophysiology, 236
Behavioral patterns in *Mus musculus,* 128
Beitinger, T. L. Diel activity rhythms and thermoregulatory behavior of bluegill in response to unnatural photoperiods, 96
Bell gills, in embryonic development, 480
Berg, C. J., Jr. A comparison of adaptive strategies of predation among naticid gastropods, 420
Bernard, G. D. and R. Wehner. Dichroism, birefringence and structural twist in polarized-light detectors of insects, 421
Beswick, D. See C. Markowitz, 435
Bioassay, for juvenile hormone, 506
Biogeographic, island theory, 440
Biological rhythms, in phytoplankton, 408
Bioluminescence spectra, by photosensitive devices, 443
Biomechanics, in water pumping, in *Chaetopterus,* 136
Biotic census, Cape Cod Bay, 287

Birefringence, in insects, 421
  in squid giant axons, 444
  optical analysis of, 421
Bishop, S. H. See L. Cooley, 424
Blake, J. A. and K. H. Woodwick. Reproduction and larval development of *Pseudopolydora paucibranchiata* (Okuda) and *Pseudopolydora kempi* (Southern) (Polychaeta: Spionidae), 109
Bleiweiss, R. See T. J. M. Schopf, 446
Blood-feeding requirements, mosquito, 384
Blood pressure, in *Pomotamus saltatrix,* 425
Bluegills, diel activity rhythms in, 96
  thermoregulatory behavior in, 96
Blue-green alga, gas vacuole formation in, 448
  turgor pressure changes in, from photosynthate, 428
Bohr, V. See R. Llinas, 435
Bone, Q. and G. O. Mackie. Skin impulses and locomotion in *Oikopleura* (Tunicata: Larvacea), 267
Brown, J. A. Sidereal-day variation in spontaneous activity of the mouse, *Mus musculus,* 128
Brown, J. E., L. B. Cohen, P. DeWeer, L. H. Pinto, W. N. Ross, and B. M. Salzberg. Arsenazo III, an indicator for rapid changes of intracellular ionized calcium in squid giant axons, 421
Brown, J. E. See L. H. Pinto, 441 and G. Waloga, 449
Brown, S. C. Biomechanics of water-pumping by *Chaetopterus variopedatus* Renier. Skeletomusculature and kinematics, 136
α-Bungarotoxin, in sperm cell function, 438
Burdick, C. J. See B. A. Taylor, 447
Busycon, sperm, conformational changes in, 429
Busycotypus, egg production in, 443
Byrd, E. W., Jr. The block to polyspermy in sea urchin fertilization: an analysis with agents affecting the cell surface, 422

C

Caeca, of isopods, 348
Calanoids, in Gulf Stream, 492
Calcium, and aequorin luminescence in amoebae, 448
  in squid axon, 421
  ultrastructural localization of, in *Arbacia* eggs, 446
Calcium ionophore, in sperm block of *Arbacia* eggs, 449
Calder, D. R. Biotic census of Cape Cod Bay: hydroids, 287
Cape Cod Bay, hydroids, 287
Capitella capitata, uptake of primary amines in, 397
Cardasis, C. See H. Schuel, 446
Carniol, P. See A. B. DuBois, 425
Carotenoids, 178
Case, J. F. See P. A. V. Anderson, 80
Castrucci, A. M. de L., and E. G. Mendes. Ultrastructure of the pigmentary system and chromatophorotropic activity in land isopods, 467
Cavanaugh, C. M. Observations on mating behavior in *Limulus polyphemus,* 422
Cellular nature, of pigment, in *Ecteinascidia turbinata,* 178
Cellulases, in isopods, 348
Cement gland, in cirripeds, 316
Chaetopterus, eggs, radiation of, 449
  oocyte maturation in, 430
  water pumping in, 136
Chamaecyparis, sediment deposition, growth irregularities in, 434
Chambers, E. L. Potassium exchange in unfertilized sea urchin (*Arbacia punctulata*) eggs, 422
Charlton, J. S. See A. Fein, 426
Childress, J. J. See Z. M. Fuzessery, 522
Chippendale, G. M. and C.-M. Yin. Reappraisal of proctodone involvement in the hormonal regulation of larval diapause, 151
Chlorophyll, in Gulf Stream, 492
Chemosensitivity, crustaceans, 522
Chromatophores, in isopods, 467
  in *Palaemonetes,* 165
Ciliary length, in *Arbacia,* 444
Cirripedia, tissue cultures, 316
Clark, T. G. and H. Kanatani. Effect of some inhibitors of proteolytic enzymes on maturation of starfish oocytes, 423
Clibanarius vittatus, ecology of, 205
Cloud, J. G. and A. W. Schuetz. Spontaneous maturation of isolated ovarian follicles from the starfish (*Asterias forbesi*): Interaction of pH, Ca^{+2} and follicle cells, 423
Cnidaria, hydroids of Cape Cod Bay, 287
Cochran, R. C. and H. Kanatani. Site of production of maturation-inducing substance in the starfish testis, 424
Coelenterate, extrusion of algal symbionts from, 590
Cohen, L. B. See J. E. Brown, 421; W. N. Ross, 444; and B. M. Salzberg, 445
Colchicine, in erythrophores of *Palaemonetes,* 165
Collins, T. See G. Weissmann, 450

Colonial behavior, in *Renilla kollikeri,* 80
Color change, in isopods, 467
Community stability, in tundra ponds, 408
Concanavalin-A and ciliary length, in *Arbacia,* 444
Conformational changes in *Busycon* and *Goniobasis* sperm, 429
CONRAD, J. E. See W. G. EGAN, 492
COOLEY, L., D. R. CRAWFORD, and S. H. BISHOP. Urease from the lugworm *Arenicola cristata,* 424
Corpora allata and metamorphosis, 214
Cortex, sperm, 551
COSTLOW, J. D. See U. E. H. FYHN, 316
Crab larvae, polarotaxis in, 251
CRAWFORD, D. R. See L. COOLEY, 424
Crayfish, water displacement and orientation, 451
Cricosphaera carterae, glycine uptake in, 425
Crustacea, bathypelagic, 522
  chemosensitivity, 522
  hormone activity in, 165
  larvae, 251
  littoral, 522
Cryptocotyle, cercariae emergence of, 446
Crystal property, of larval spicule, of *Arbacia,* 431
Cultivation, of nudibranch species, 539
Culture media, of cirripeds, 316
Cyanidium caldarium, δ-aminolevulinic acid in, 432
Cycle, mean sidereal-day, in *Mus musculus,* 128
Cyclic gill movement, in *Limulus,* 452
Cyst, in zooxanthella, 590
Cytochalasin B, in erythrophores of *Palaemonetes,* 165
Cytological changes, in larval development, 151

D

DE ALBUJA, C. M. See E. M. DEL PINO, 480
DEL PINO, E. M., M. L. GALARZA, C. M. DE ALBUJA, AND A. A. HUMPHRIES, JR. The maternal pouch and development in the marsupial frog *Gastrotheca riobambae* (Fowler), 480
Depolymerization, microtubule, of mitotic spindle, 433
Desiccation tolerance, in *Melampus,* 442
Development, of grubby, 373
DEWEER, P. See J. E. BROWN, 421
DFPase, in squid nerve, 429
Diatraea grandiosella, larval diapause in, 151
Dichroism, in insects, 421
  in rhabdomeric photoreceptor, 427
  in squid giant axons, 444
  optical analysis of, 421
Diel activity rhythms, in bluegill, 96
Differentiation, of enzymes in ascidian eggs, 451
Digestive physiology, of isopods, 348
Dinoflagellate, in zooxanthella, 590
Diptera, sexuality in, 365
DISPEZIO, M. See H. W. STUNKARD, 447
Diurnal rhythms, in *Uca pugnax,* 432
DUBOIS, A. B., P. CARNIOL, AND R. D. B. MORRIS. Effect of gravity and exercise on the heart rate and blood pressure of bluefish (*Pomotamus saltatrix*), 425
DUCKWORTH, D. AND A. FARMANFARMAIAN. Uptake of glycine by the coccolithophorid *Cricosphaera carterae,* a unicellular marine alga, 425
DUNHAM, P. B. See G. WEISSMANN, 450
DUTTON, A. R. AND T. J. M. SCHOPF. Morphologic differentiation can approximately parallel genetic differentiation in the ectoproct *Schizoporella errata* in coastal populations from Woods Hole to Orleans, 425

E

β-ecdysone infusion, in *Manduca sexta* larvae, 568
Echinochrome, in *Arbacia,* 441
Ecology, of *Ptychodera flava,* 455
  zooplankton, 492
Ecteinascidia, pigmentation in, 178
EDMAN, J. D. See G. F. O'MEARA, 384
EGAN, W. G. AND J. E. CONRAD. Summer abundance and ecology of zooplankton in the Gulf Stream, 492
Egg production, *Busycotypus,* 443
  in mosquitoes, 384
Electrophysiology, in medusae of *Proboscidactyla,* 236
  in *Renilla kollikeri,* 80
  of lagena function, 438
  of *Proboscidactyla,* 236
Emerita asiatica, hemocytes of, 226
Embryogenesis, in *Laeonereis culveri,* 186
Embryology, of *Gastrotheca riobambae,* 480
  of grubby, 373
  of *Nassarius,* 580
  of *Phestilla,* 539
Energy coupling, in toadfish, 420
Enzymes, histospecific, in ascidian eggs, 451
EPEL, D. See G. PERRY, 441
Epifauna, marine communities, 440
Epithelial conduction, in *Prosboscidactyla,* 236
Erythrophores, ovarian, integumentary in *Palaemonetes,* 165
EVERS, A. See G. WEISSMANN, 450

Extracellular matrix, ultrastructure, 419
Extrusion of algal symbionts, from coelenterate, 590

F

Fain, M. J. and L. M. Riddiford. Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L.), 506
Farmanfarmaian, A. See D. Barklow, 420, and D. Duckworth, 425
Fatigue phenomena, in *Prosboscidactyla*, 236
Feeding behavior, crustacean, 522
  of *Laeonereis culveri,* 186
Fein, A. and J. S. Charlton. Local membrane currents in *Limulus* ventral photoreceptors, 426
Fertilization, in *Arbacia,* 446
  in *Hydrolimax grisea,* 551
Fingerman, M., S. W. Fingerman and D. T. Lambert. Colchicine, cytochalasin B, and pigment movements in ovarian and integumentary erythrophores of the prawn, *Palaemonetes vulgaris,* 165
Fish, hemoglobins, subunit interactions of, 435
Fisher, M. See T. A. Lyerla, 178
Fitzgerald, M. P. See J. E. Jurgenson, 432
Flagella, biflagellate spermatozoa, 551
Fluorescamine, 397
Fluorescence microscopy, during larval diapause, 151
Food webs, in salt marsh, 435
Forward, R. B., Jr. See S. E. Via, 251
Fuzessery, Z. M. and J. J. Childress. Comparative chemosensitivity to amino acids and their role in the feeding activity of bathypelagic and littoral crustaceans, 522
Fyhn, U. E. H. and J. D. Costlow. Tissue cultures of cirripeds, 316

G

Galarza, M. L. See E. M. del Pino, 480
Gamete release, in sea cucumber, 433
Gastrotheca riobambae, reproduction in, 480
Gas vacuole, formation, in blue-green alga, 448
Gelperin, A. An identified serotonergic input has reciprocal effects on two electrically coupled motoneurons in the terrestrial slug, *Limax maximus,* 426
Genetic differentiation of *Schizoporella errata,* 425
Genetics of mosquito egg production, 384
Glucagon, from proglucagon, in angler-fish, 439
Glycine, in toadfish intestine, 420
  uptake in *Cricosphaera carterae,* 425
Gnathophausia ingens, 522
Gold, K. and E. A. Morales. Studies on Woods Hole Tintinnida using scanning electron microscopy, 427
Goldsmith, T. H. and R. Wehner. Photoinduced dichroism in a rhabdomeric photoreceptor: evidence for restricted rotation of pigment molecules, 427
Goldsmith, T. H. See R. Wehner, 450
Goniobasis, sperm, conformational changes in, 429
Govind, C. K. and F. Lang. Synaptic connection between medial giant axons in the lobster, *Homarus americanus,* 428
Grant, N. G. and A. E. Walsby. The contribution of photosynthate to turgor pressure changes in a gas vacuolate blue-green alga, 428
Granular hemocyte, in *Emerita asiatica, 226*
Green, D. J. See D. B. Sattelle, 445
Grinvald, A. See W. N. Ross, 444
Growth, differences in *Pseudopolydora* species, 109
  irregularities in *Chamaecyparis,* 434
  rate regulation, 408
Grubby, early development in, 373
Gut structure, of isopods, 348

H

Half-life, of JH, in final instar larvae, 568
Hamby, R. J. Heat effects on a marine snail, 331
Harris, L. G. Studies on the life history of two coral-eating nudibranchs of the genus *Phestilla,* 539
Haschemeyer, A. See J. Nielsen, 439
Hassall, M. and J. B. Jennings. Adaptive features of gut structure and digestive physiology in the terrestrial isopod *Philoscia muscorum* (Scopoli) 1763, 348
Hatching substance, production, in *Nassarius,* 580
Heart rate in *Pomotamus saltatrix,* 425
Heat coma, in *Littorina littorea,* 331
Hemichordate, ecology and respiration, 455
Hemocytes, of *Emerita asiatica,* 226
Hemoglobins, fish, subunit interactions of, 435
Hemolymph, JH titers in, 506
Henley, C. Conformational changes in chromatin of spermatozoa of two prosobranch molluscs (*Goniobasis* and *Busycon*), 429
Herman, L. See H. Schuel, 446
Hermit crab, shell occupation by, 205

HEUSER, J. E. AND T. S. REESE. Structural changes at the "active zones" of synapses in stimulated *Torpedo* electric organs, 429
Hirudo, molecular probes, optical recordings in, 445
Histone acetylation, in *Arbacia* development, 447
Homarus, medial giant axon in, 428
Homosexuality, in mosquitoes, 365
Hormonal control, of larval diapause, 151
Hornet venom, on squid axon membranes, 440
HOSKIN, F. C. G. AND E. R. KORDIK. Rhodanese and DFPase in relation to isetheionate in squid nerve, 429
House-rudiment expansion, in *Oikopleura,* 267
HUMPHRIES, A. A., JR. See E. M. DEL PINO, 480
HUMPHREYS, T., W. YONEMOTO, S. HUMPHREYS AND D. ANDERSON. Purification of *Microciona prolifera* aggregation factor, 430
Hydrogen ion concentration, and hemocytes, 226
Hydroids, of Cape Cod Bay, 287
Hydrolimax grisea, ultrastructure of sperm in, 551
Hydrology, Gulf Stream, 492
Hydrozoa, of Cape Cod Bay, 287

I

IKEGAMI, S., T. S. OKADA AND S. S. KOIDE. Factors involved in oocyte maturation in the polychaete, *Chaetopterus pergamentaceus,* 430
IKEGAMI, S. See S. S. KOIDE, 433
ILAN, J. AND J. ILAN. Studies on the mechanism of the initiation of polypeptide synthesis in sea urchin embryos *in vivo,* 431
Ileum, histology of larval and pupal, 151
Induction of metamorphosis, in *Phestilla,* 539
Infection by algal symbionts, in coelenterates, 590
INOUÉ, S. AND K. OKAZAKI. Crystal property of larval spicule of *Arbacia punctulata,* 431
INOUÉ, S. See D. P. KIEHART, 433
Integrative processes, in *Renilla* colony, 80
Interspecific shell fights, by hermit crabs, 205
Intracellular recordings, of squid, 441
Ionic regulation, in squid brain, 442
Insects, juvenile hormone in, 568
Isetheionate, in squid nerve, 429
ISMAIL, M. M. See J. AZARIAH, 455
Isopods, digestive physiology of, 348
  marine and land, 467

J

JENNINGS, J. B. See M. HASSALL, 348
JONES, J. C. Nonheterosexual behavior in mosquitoes, 365
JURGENSON, J. E., S. I. BEALE, R. R. TROXLER, M. M. BARTOLF, M. P. FITZGERALD, J. RAMUS AND J. A. SCHIFF. Biosynthesis of δ-aminolevulinic acid in *Cyanidium caldarium* and other algae, 432
Juvenile hormone, and metamorphisis, 214
  in *Manduca sexta,* 506
  in *Manduca sexta,* 568

K

KANATANI, H. See T. G. CLARK, 423, R. C. COCHRAN, 424, and S. S. KOIDE, 433
KELLY, C. J., JR. Shifts in tidal and diurnal rhythms of color change of *Uca pugnax* due to burrow location, 432
KIEHART, D. P. AND S. IONUÉ. Microtubule depolymerization in local regions of the mitotic spindle by Ca^{++} micro-injection, 433
KIEHART, D. See C. MARKOWITZ, 435
Kinematics, in *Chaetopterus,* 136
KOHN, N. See W. N. ROSS, 444
KOIDE, S. S., S. IKEGAMI AND H. KANATANI. Gamete release from isolated sea cucumber gonads by 1-methyladenine, 433
KOIDE, S. S. See S. IKEGAMI, 430
KORDIK, E. R. See F. C. G. HOSKIN, 429

L

Laboratory-rearing, of *Laeonereis culveri,* 186
  of nudibranchs, 539
LADERMAN, A. D. Sediment depositions and growth irregularities in *Chamaecyparis thyoides* in kettle bogs, 434
Laeonereis culveri, larval development of, 186
Lagena, function, electrophysiology of, 438
LAMBERT, D. T. See M. FINGERMAN, 165
LANDOWNE, D. Sodium efflux from voltage clamped squid axons, 434
LANG, F. See C. K. GOVIND, 428
LANGLEY, K. H. See D. B. SATTELLE, 445
Larvae, of *Laeonereis culveri,* 186
  of *Manduca sexta,* 506
  of *Nassarius,* 580
Larval development, polarotaxis in, 251
  in *Manduca sexta,* 214, 506, 568
  in *Pseudopolydora,* 109
Larval diapause, hormonal regulation in, 151
Larval spicule, crystal property, in *Arbacia,* 431
Laser light scattering, neurosecretory tissue, 445

Lecithotrophic development, in *Phestilla sibogae,* 539
Lee, J. J., J. H. Tietjen and C. Mastropaolo. Trophic relationships at lower and intermediate steps in a salt marsh detritus-based food web, 435
Lepomis macrochirus, behavior of, 96
Life cycles, of *Phestilla,* 539
Ligia exotica, ultrastructure of, 467
Limax. motoneurons, serotonergic input in, 426
Limiting nutrient, 408
Limulus, cyclic gill movement in, 452
  mating behavior in, 422
  photoreceptors, arsenazo III on, 449
  photoreceptor membrane currents, 426
Liposomes, arsenazo III in, 450
Littorina littorea, and *Cryptocotyle,* 446
  temperature acclimation in, 331
Liver elongation factor 1, in toadfish, 439
Llinas, R., K. Walton, and V. Bohr. The action of 3- and 4-aminopyridine on synaptic transmission in the squid giant synapse, 435
Locality-dependent, alleles, in *Modiolus,* 446
Locomotion, in *Oikopleura,* 267
  of bluegill, 96
Lorand, L. See W. Troll, 449
Luminescence, in *Renilla kollikeri,* 80
Lund, W. A., Jr. and B. C. Marcy, Jr. Early development of the grubby, *Myoxocephalus aenaeus* (Mitchill), 373
Lyerla, T. A., J. H. Lyerla and M. Fisher. Pigmentation in the orange tunicate, *Ecteinascidia turbinata,* 178
Lysosomes, in larval diapause, 151

M

Mackie, G. O. See Q. Bone, 267
Manduca sexta, larval development, 506
  metamorphosis in, 214, 568
Mantle tissue, in *Ecteinascidia turbinata,* 178
Marcy, B. C., Jr. See W. A. Lund, Jr., 373
Marine annelids, amines in, 397
Markey, C. H. See G. A. Wyse, 452
Markowitz, C., D. Beswick, D. Kiehart and D. Powers. Subunit interactions of fish hemoglobins, 435
Marsupial frog, reproduction, 480
Mastropaolo, C. See J. J. Lee, 435
Maternal pouch, in *Gastrotheca riobambae,* 480
Mating behavior, in *Limulus,* 422
Maturation-inducing substance, in starfish testis, 424
Mazurkiewicz, M. Larval development and habits of *Laeonereis culveri* (Webster) (Polychaeta: Nereidae), 186
McMahon, R. F. and W. D. Russell-Hunter. Temperature and salinity effects on *Melampus bidentatus* Say with special reference to oxygen uptake rates and survival, 436
Mechanoreceptors, crayfish, 451
Medial giant axons, in *Homarus,* 428
Medusoid phase, in hydromedusae, 236
Metamorphosis, in *Manduca sexta,* 214, 568
  in *Pseudopolydora,* 109
  threshhold size for, 214
Metarhodopsin, in rhabdomeric photoreceptor, 450
1-methyladenine, in sea cucumber gamete release, 433
Melampus, behavior, physiology in, 442
  temperature, salinity on respiration, 436
Membrane, changes in *Tetrahymena,* 419
  currents in *Limulus* photoreceptors, 426
  liposomes, and arsenazo III, 450
  squid, axon, hornet venom on, 440
  whorls in sperm, 551
Mendes, E. G. See A. M. de L. Castrucci, 467
Microciona prolifera, aggregation factor in, 430
Microciona, ultrastructure, 419
Micromeres, in spicule formation, in *Arbacia* embryos, 439
Microtubule depolymerization, of mitotic spindle, 433
Microtubules, in *Palaemontes,* 165
Mitchell, K. A. An analysis of shell occupation by two sympatric species of hermit crab. I. Ecological factors, 205
Mitchell, S. W. Variation in the ontogenetic development of radial ornamentation in pelecypods and brachiopods, 437
Mitotic spindle, microtubule depolymerization in, 433
Modiolus, alleles in, 446
Molecular probes, in leech, 445
Morales, E. A. See K. Gold, 427
Morgan, K. Effects of pentobarbital on the squid giant synapse, 437
Morphology, of hemocytes, in *Emerita asiatica,* 226
Morris, R. D. B. See A. B. DuBois, 425
Mosquitoes, autogenous egg production in, 384
  sexual behavior in, 365
Motlility, sperm cell, 551
Motoneurons, *Limax,* serotonergic input in, 426
Motor activity, spontaneous, in *Mus musculus.* 128
  in *Oikopleura,* 267

Mus musculus, behavioral patterns in, 128
Myoxocephalus aenaeus, development in, 373

N

Najib, M. A. See J. Azariah, 455
Narahashi, T. See J. L. Parmentier, 440
Narins, P. M. Electrophysiological determination of the function of the lagena in terrestrial amphibians, 438
Naticid gastropods, predation strategies, 420
Natural history, of *Pseudopolydora,* 109
Natural primary amines, net flux of, 397
Nelson, L. α-Bungarotoxin blockade of sperm cell function, 438
Nereid polychaetes, 186
Nereis diversicolor, uptake of primary amines in, 397
Nervous system, of marine snails, 331
Nervous tissues, particle motion in, 445
Neurohormones, in *Busycotypus,* 443
Neurosecretory tissue and laser light, 445
Newton, W. D. The ultrastructure of the aflagellate spermatozoön of the freshwater turbellarian *Hydrolimax grisea* (Platyhelminthes: Plagiostomidae), 551
Nielsen, J. and A. Haschemeyer. Distribution and multiple activities of liver elongation factor 1 in temperature acclimated toadfish, 439
Nijhout, H. F. A threshhold size for metamorphosis in the tobacco hornworm, *Manduca sexta* (L.), 214
Nijhout, H. F. Dynamics of juvenile hormone action in larvae of the tobacco hornworm, *Manduca sexta* (L.), 568
Noe, B. D. and G. E. Bauer. Identification of an intermediate in the metabolic conversion of proglucagon to glucagon, 439
Nonheterosexuality, in mosquitoes, 365
Nuclear condensation, in sperm, 551
  fibers, in sperm, 551
Nucleus, sperm, 551
Nudibranchs, coral-eating, 539
Nutrient bioassay, 408

O

Odhneria, developmental stages of, 447
Ohman, M. D. See T. J. M. Schopf, 446
Oikopleura, locomotion in, 267
Okada, T. S. See S. Ikegami, 430
Okazaki, K. See S. Inoué, 431
Okazaki, K. Spicule formation by micromeres isolated from embryos of *Arbacia punctulata,* 439
O'Meara, G. F. and J. D. Edman. Autogenous egg production in the salt-marsh mosquito, *Aedes taeniorhynchus,* 384
Ontogeny, of polarotaxis, in larval development, 251
Oocyte maturation, in *Chaetopterus pergamentaceus,* 430
Optical recordings, in leech, 445
Operculum, in *Nassarius* eggs, 580
Organ culture, of cirripeds, 316
Ostrinia nubilalis, larval diapause in, 151
Osman, R. W. Island biogeographic theory as a major principle for understanding marine epifaunal communities, 440
Ovarian follicles, starfish, spontaneous maturation in, 423
Ovary, in cirripeds, 316

P

Pagurus longicarpus, ecology of, 205
Palaemonetes, erythrophores of, 165
Parabiosis, in larvae of *Manduca sexta,* 568
Parasites, trematode, of birds, 447
Parmentier, J. L. and T. Narahashi. Effects of hornet venom on squid axon membranes, 440
Pattern, sidereal-day in *Mus musculus,* 128
Pechenik, J. A. The escape of veligers from the egg capsules of *Nassarius obsoletus* and *Nassarius trivittatus* (Gastropoda, Prosobranchia), 580
Pennatulids, colonial behavior and luminescence in, 80
Pentobarbital, on squid giant synapse, 437
Periodicity, sidereal-day, in *Mus musculus,* 128
Perry, G. and D. Epel. Calcium control of a cyanide insensitive respiration in *Arbacia punctulata* eggs, and its relation to echinochrome, 441
Phestilla, life history, 539
Philoscia, gut structure and digestive physiology in, 348
Phosphate, and photosynthesis, 408
Photoperiods, and bluegill behavior, 96
Photoproduct, tryptophan, antigrowth action, 452
Photoreceptors, intracellular recordings of, 441
  Limulus, arsenazo III on, 449
  Limulus, membrane changes in, 426
  rhabdomeric, metarhodopsin in, 450
Photosensitive devices, for bioluminescence spectra, 443
Photosynthate, in turgor pressure changes, blue-green alga, 428
Photosynthetic rhythms, in phytoplankton, 408

Physiology, of molluscan nervous system, 331

Phytoplankton, rhythms in photosynthetic rates of, 408

Pigment, in *Ecteinascidia turbinata,* 178
  migration in erythrophores of *Palaemonetes,* 165
  of isopods, 467
  in *Laeonereis culveri,* 186

Pinto, L. H. and J. E. Brown. Intracellular recordings from photoreceptors of the squid retina, 441

Pinto, L. H. See J. E. Brown, 421, and G. Waloga, 449

Planktotrophic development in *Phestilla melanobranchia,* 539

Polarotaxis, in crab larvae, 251

Pollard, H. B. and J. L. Barker. Ionic regulation of transmitter uptake in squid brain synaptosomes, 442

Polychaete, oocyte maturation in, 430
  water pumping in, 136

Polypeptide synthesis, sea urchin, 431

Polypoid phase, in hydromedusae, 236

Polyspermy, block to, 422

Pomotamus saltatrix, heart rate in, 425

Population distribution, in Gulf Stream, 492

Potassium exchange, in *Arbacia* eggs, 422

Pouch, of *Gastrotheca,* 480

Powers, D. See C. Markowitz, 435

Predation strategies, in naticid gastropods, 420

Price, C. H. and W. D. Russell-Hunter. Behavioral and physiological aspects of water relations in the high littoral snail, *Melampus bidentatus* Say, 442

Primary amines, uptake of in marine annelids, 397

Proboscidactyla flavicirrata, behavior and electrophysiology of, 236

Proctodone, in regulation of larval diapause, 151

Proglucagon, to glucagon, in angler-fish, 439

Prosobranch physiology, 331

Proteolytic enzymes, inhibitors of, in starfish oocytes, 423

Pseudopolydora, reproduction and larval development in, 109

Ptychodera flava, ecology and respiration in, 455

Pumping activity, muscle action in, *Chaetopterus,* 136
  in *Oikopleura,* 267

Purification, of aggregation factor, in *Microciona prolifera,* 430

R

Radiation, and *Chaetopterus* eggs, 449

Ram, J. L. Laying of egg capsules in *Busycotypus* induced by nervous system extracts, 443

Ramus, J. See J. E. Jurgenson, 432

Ravindranath, M. H. Effects of hydrogen ion concentration on the morphology of hemocytes of the mole-crab *Emerita asiatica,* 226

Receptors, antennule and dactyl sensitivity in crustaceans, 522

Reese, T. S. See J. E. Heuser, 429

Rehydration, in *Melampus,* 442

Renilla kollikeri, colonial behavior and luminescence in, 80

Reproduction, *Gastrotheca riobambae,* 480
  in *Laeonereis culveri,* 186
  in *Nassarius,* 580
  in Pseudopolydora, 109

Respiration, in *Limulus,* 452
  Melampus, temperature, salinity on, 436
  Ptychodera flava, 455

Revel, J.-P. See W. S. Adair, 419

Reynolds, G. T. Improved photosensitive devices for observing *in vivo* bioluminescence spectra, 443

Reynolds, G. T. See D. L. Taylor, 448

Rhabdomeric photoreceptor, dichroism in, 427
  metarhodopsin on, 450

Rhithropanopeus harrisi, larvae of, 251

Rhodanese, in squid nerve, 429

Rhythmic activity, Larvacea, 267

Riddiford, L. M. See M. J. Fain, 506

Riederer-Henderson, M. A. and J. L. Rosenbaum. Increase in ciliary length of *Arbacia* blastulae by Concanavalin-A or trypsin treatment, 444

Rosenbaum, J. L. See M. A. Riederer-Henderson, 444

Ross, W. N., L. B. Cohen, B. M. Salzberg, N. Kohn, and A. Grinvald. Extrinsic birefringence and dichroism changes in squid giant axons, 444

Ross, W. N. See J. E. Brown, 421, and B. M. Salzberg, 445

Russell-Hunter, W. D. See R. F. McMahon, 436, and C. H. Price, 442

Rustad, R. C. See G. Viswanathan, 449

S

Salinity, on *Melampus* respiration, 436
  on respiration in *Ptychodera flava,* 455

Salt-marsh mosquitoes, autogeny in, 384

Salt marsh, trophic relationships in, 435

Salzberg, B. M., L. B. Cohen, W. N. Ross, A. S. Waggoner and C. H. Wang. New and more sensitive molecular probes of membrane potential: simultaneous optical

recordings from several cells in the central nervous system of the leech, 445
Salzberg, B. M. See J. E. Brown, 421, and W. N. Ross, 444
Sattelle, D. B., D. J. Green and K. H. Langley. Laser light scattering from a neurosecretory tissue, 445
Scanning electron microscopy, of Tintinnida, 427
Schiff, J. A. See J. E. Jurgenson, 432
Schizoporella errata, genetic differentiation in, 425
Schopf, T. J. M., M. D. Ohman, and R. Bleiweiss. Significant age-dependent and locality-dependent changes occur in gene frequencies in the ribbed mussel *Modiolus demissus* from a single salt marsh, 446
Schopf, T. J. M. See A. R. Dutton, 425
Schuel, H., C. Cardasis, L. Herman, and W. L. Wilson. Ultrastructural localization of calcium in the early stages of fertilization in *Arbacia* eggs, 446
Schuel, H. See W. Troll, 449
Schuetz, A. W. See J. G. Cloud, 423
Sea anemone, 590
Sea cucumber, gamete release in, 433
Sea urchin, ciliary length in, 444
  crystal property of larval spicule in, 431
  eggs, potassium exchange in, 422
  eggs, respiration in, 441
  eggs, sperm block in, 449
  eggs, ultrastructure of, 446
  embryos, spicule formation in, 439
  fertilization, 422
  histone acetylation of, 447
  polypeptide synthesis in, 431
Sediment cores, artificial, 397
Sediment deposition, in *Chamaecyparis,* 434
Sensory physiology, Larvacea, 267
Serotonergic input, in *Limax* motoneurons, 426
Sexual behavior, mosquitoes, 365
Shell selection, by hermit crabs, 205
Sidereal variation, in *Mus musculus,* 128
Sinus gland, in isopods, 467
Skeletomusculature, in Chaetopterus, 136
Skin impulses, in *Oikopleura,* 267
Smooth-muscle physiology, 331
Sodium efflux, in squid axons, 434
Somatic size and molting in *Manduca sexta,* 214
Species numbers, in epifaunal communities, 440
Spectral sensitivity, of polarotaxis, in crab larvae, 251
Spencer, A. N. Behavior and electrical activity in the hydrozoan *Proboscidactyla flavicirrata* (Brandt). II. The medusa, 236
Spermatozoön, ultrastructure of, 551
Sperm block, in *Arbacia* eggs, 438, 449
Spicule formation, in *Arbacia* embryos, 439
Spionid polychaetes, larval development in, 109
Spontaneous maturation, starfish ovarian follicles, 423
Squid, axon membrane, hornet venom on, 440
  axons, birefringence, dichroism in, 444
  axons, sodium efflux in, 434
  brain, synaptosomes, 442
  giant axon, 421
  giant synapse, 437
  photoreceptors of retina, 441
  synaptic transmission in, 435
Starfish oocytes, proteolytic enzyme inhibitors in, 423
  ovarian follicles, spontaneous maturation in, 423
  testis, maturation-inducing substance in, 424
Steele, R. D. Stages in the life history of a symbiotic zooxanthella in pellets extruded by its host *Aiptasia tagetes* (Duch. and Mich.) (Coelenterata, Anthozoa), 590
Stephens, G. C. Uptake of naturally occurring primary amines by marine annelids, 397
Stross, R. G. Causes of daily rhythms in photosynthetic rates of phytoplankton. II. Phosphate control of expression in tundra ponds, 408
Stryeski, K. The effect of light intensity and temperature on the emergence of *Cryptocotyle lingua* cercariae from the host, *Littorina littorea,* 446
Stunkard, H. W. and M. DiSpezio. Studies on trematode parasites of birds: developmental stages of *Odhneria* sp., (Microphallidae), 447
Substrate film method for cellulases, 348
Substrate selection, by hermit crabs, 205
Subunit interactions, of fish hemoglobins, 435
Synapse, giant, squid, 437
  Torpedo, active zones, 429
  transmission, in squid, 435
Synaptosomes, in squid brain, 442

T

Taylor, B. A. and C. J. Burdick. Histone acetylation during the early stages of sea urchin (*Arbacia punctulata*) development, 447
Taylor, D. L., G. T. Reynolds, and R. D. Allen. Detection of free calcium ions in amoebae by aequorin luminescence, 448

Temperature, acclimation in marine snails, 331, 436
  preference in bluegills, 96
  on *Melampus* respiration, 436
  regulation of bluegills, 96
Tetrahymena, membrane changes in, 419
Thermoregulatory fish activity, 96
Tidal rhythms, in *Ptychodera flava,* 455
  in *Uca pugnax,* 432
TIETJEN, J. H. See J. J. LEE, 435
Tintinnida, scanning electron microscopy of, 427
Tissue cultures, of cirripeds, 316
Toadfish, energy coupling in, 420
  temperature acclimation in, 439
Torpedo, synapses, active zones of, 429
Transmission, of zooxanthella, 590
Transmitter uptake, ionic regulation of, 442
TRIPLETT, B. A. AND A. E. WALSBY. Measurement of gas vacuole formation rates in a blue-green alga, 448
TROLL, W., H. SCHUEL, AND L. LORAND. Calcium ionophore A23187 induced block to sperm penetration of *Arbacia* eggs is prevented by protease inhibitors, 449
Trophic relationships in salt marsh, 435
TROXLER, R. R. See J. E. JURGENSON, 432
Trypsin treatment, and ciliary length in *Arbacia,* 444
Tryptophan, photoproduct, antigrowth action, 452
Tube-dwelling worm, behavior in, 136
Tunicate, pigment of, 178
Turgor pressure changes, in blue-green alga, from photosynthate, 428
Typhlosole, in isopods, 348

U

Uca pugnax, tidal, diurnal rhythms in, 432
Urease, in *Arenicola cristata,* 424
Ultrastructure, in *Ecteinascidia,* 178
  in isopods, 467
  sperm, of *Hydrolimax grisea,* 551
Uptake of primary amines, in marine annelids, 397
Urochordata, 178

V

Veliger, cultivation of *Phestilla,* 539
  escape from egg capsules, of *Nassarius,* 580
Vespula, toxin of, 440
VIA, S. E. AND R. B. FORWARD, JR. The ontogeny and spectral sensitivity of polarotaxis in larvae of the crab *Rhithropanopeus harrisi* (Gould), 251,
VISWANATHAN, G. AND R. C. RUSTAD. Radiation-induced cleavage delay in *Chaetopterus* eggs, 449

W

WAGGONER, A. S. See B. M. SALZBERG, 445
WALOGA, G., J. E. BROWN, AND L. H. PINTO. Detection of changes of Ca(in) from *Limulus* ventral photoreceptors using arsenazo III, 449
WALSBY, A. E. See N. G. GRANT, 428, and B. A. TRIPLETT, 448
WALTON, K. See R. LLINAS, 435
WANG, C. H. See B. M. SALZBERG, 445
Water displacement, in crayfish, 451
Water pumping, in Polychaetes, 136
Water relations, in *Melampus,* 442
WEHNER, R. AND T. H. GOLDSMITH. Restrictions on translational diffusion of metarhodopsin in the membranes of a rhabdomeric photoreceptor, 450
WEHNER, R. See G. D. BERNARD, 421, and T. H. GOLDSMITH, 427
WEISSMANN, G., T. COLLINS, A. EVERS AND P. DUNHAM. Membrane perturbation: studies employing a calcium-sensitive dye (arsenazo III) in liposomes, 450
WHITTAKER, J. R. Differentiation of two histospecific enzymes in the same cleavage-arrested ascidian egg, 451
WIESE, K. On the mechanism of orientation towards water displacement sources in crayfish, 451
WILSON, W. L. See H. SCHUEL, 446
WOODWICK, K. H. See J. A. BLAKE, 109
WYSE, G. A. AND C. H. MARKEY. The effect of forced cyclic gill movement on the respiratory rhythm of *Limulus,* 452

X

X-organ, in isopods, 467

Y

YIN, C.-M. See G. M. CHIPPENDALE, 151
YONEMOTO, W. See T. HUMPHREYS, 430
YULO, T. See S. ZIGMAN, 452

Z

ZIGMAN, S. AND T. YULO. Subcellular localization of the antigrowth action of a purified sunlight-induced tryptophan photoproduct, 452
Zoeal stages, of larval crabs, 251
Zooplankton distribution, Gulf Stream, 492
Zoosporagium and zoospores of zooxanthella, 590

Continued from Cover Two

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E. We strongly recommend that more unusual words in journal titles be spelled out in full, rather than employing lengthy, peculiar "abbreviations" or new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation. Even in more familiar languages, *Z. Vererbungslehre* is preferred to *Z. VererbLehre* (World List) or *Z. Vererbungsl.* (USASI). *Accurate and complete communication of the reference is more important than minor savings in printing costs.*

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some Biological Abstracts listings).

H. Spell out *London, Tokyo, Paris, Edinburgh, Lisbon, etc.* where part of journal title.

I. Series letters *etc.* immediately before volume number.

J. A few well-known international journals in their preferred forms rather than World List or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond.; Science, N.Y.; Evolution, Lancaster, Pa.*)

K. The correct abbreviation for The Biological Bulletin is *Biol. Bull.*

5. ***Figures.*** The dimensions of the printed page, 5 by $7\frac{3}{8}$ inches, should be kept in mind in preparing figures for publication. Illustrations should be large enough so that all details will be clear after appropriate reduction. Explanatory matter should be included separately in legends as far as possible, although the axes should always be numbered and identified on the illustration itself. Figures should be prepared for reproduction as either line-cuts or halftones; no other methods will be used. Figures to be reproduced as line-cuts should be drawn in black ink on white paper, good quality tracing cloth or plastic, or blue-lined coordinate paper; those to be reproduced as halftones should be mounted on board, and both designating numbers or letters and scale-bars should be affixed directly on the figures. We recommend that halftones submitted to us be mounted prints made at about $1\frac{1}{2}$ times the linear dimensions of the final printing desired (the actual best reductions are achieved from copy in the range from $1\frac{1}{4}$ to 2 times the linear dimensions). As regards line-blocks, originals can be designed for even greater reductions but are best in the range $1\frac{1}{2}$ to 3 times. *All* figures should be numbered in consecutive order, with no distinction between text and plate-figures. The author's name should appear on the reverse side of all figures, and the inked originals for line-blocks must be submitted for block-making.

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CONTENTS

AZARIAH, JAYAPAUL, M. MOHAMED ISMAIL, AND M. AHMED NAJIB
Investigation on the ecology and respiratory responses of the Hemichordate *Ptychodera flava* to tidal cycles and salinity changes..... 455

CASTRUCCI, ANA MARIA DE L., AND ERASMO G. MENDES
Ultrastructure of the pigmentary system and chromatophorotropic activity in land isopods.................................. 467

DEL PINO, EUGENIA M., MARIA L. GALARZA, CARMEN M. DE ALBUJA, AND A. A. HUMPHRIES, JR.
The maternal pouch and development in the marsupial frog *Gastrotheca riobambae* (Fowler)...................................... 480

EGAN, W. G., AND J. E. CONRAD
Summer abundance and ecology of zooplankton in the Gulf Stream 492

FAIN, MARGERY J., AND LYNN M. RIDDIFORD
Juvenile hormone titers in the hemolymph during late larval development of the tobacco hornworm, *Manduca sexta* (L.)......... 506

FUZESSERY, ZOLTAN M., AND JAMES J. CHILDRESS
Comparative chemosensitivity to amino acids and their role in the feeding activity of bathypelagic and littoral crustaceans........... 522

HARRIS, LARRY G.
Studies on the life history of two coral-eating nudibranchs of the genus *Phestilla*....................................... 539

NEWTON, W. DONALD
The ultrastructure of the aflagellate spermatozoon of the freshwater turbellarian *Hydrolimax grisea* (Platyhelminthes: Plagiostomidae). 551

NIJHOUT, H. FREDERIK
Dynamics of juvenile hormone action in larvae of the tobacco hornworm, *Manduca sexta* (L.)...................................... 568

PECHENIK, JAN A.
The escape of veligers from the egg capsules of *Nassarius obsoletus* and *Nassarius trivittatus* (Gastropoda, Prosobranchia)............ 580

STEELE, R. DUNBAR
Stages in the life history of a symbiotic zooxanthella in pellets extruded by its host *Aiptasia tagetes* (Duch. and Mich.) (Coelenterata, Anthozoa).. 590

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